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A genetically engineered therapeutic lectin inhibits human influenza A virus infection and sustains robust virus-specific CD8 T cell expansion

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Abstract

Seasonal influenza continues to be a global health problem. Current existing vaccines and antivirals against influenza have limited effectiveness, and typically do not stay ahead of the viral evolutionary curve. Broad-spectrum antiviral agents that are effective therapeutically and prophylactically are much needed. We have created a promising new broad-spectrum anti-influenza agent using molecular engineering of a lectin from bananas, H84T, which is well-tolerated and protective in small animal models. However, the potency and effect of H84T on human immune cells and influenza-specific immune responses are undetermined. We found that H84T efficiently inhibited influenza A virus (IAV) replication in primary human dendritic cells (DCs) isolated from blood and tonsil, preserved DC viability and allowed acquisition and presentation of viral antigen. Excitingly, H84T-treated DCs subsequently initiated effective expansion of IAV-specific CD8 T cells. Furthermore, H84T preserved the capacity of IAV-exposed DCs to present a second non-IAV antigen and induce robust antigen-specific CD8 T cell expansion. Our data support H84T as a potent antiviral in humans as it not only effectively inhibits IAV infection, but also preserves induction of robust pathogen-specific adaptive immune responses against diverse antigens, which likely is clinically beneficial.

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Author summary

Influenza causes large-scale, global morbidity and mortality. Current antiviral treatments and vaccines have significant limitations, especially when dealing with evolving strains of the virus. Banana lectin (BanLec) has broad antiviral effects on enveloped viruses, including influenza, but also causes harmful T cell proliferation and inflammation (mitogenicity). We previously used targeted molecular engineering to produce H84T BanLec (H84T), which is effective against all isolates of influenza tested and is not mitogenic. However, the effect of H84T on the human immune response to influenza had not been ascertained. Here we assessed the effect of H84T on human dendritic (antigen-presenting) cells and virus-specific T cell immune responses against influenza infection and unrelated antigen. We found that H84T treatment allowed dendritic cells to maintain their function during viral infection. H84T prevented the damage caused by viral replication and preserved the ability of dendritic cells to present not only influenza but also secondary, non-influenza antigens to T cells. Thus, H84T could be a valuable tool for controlling influenza infection and potentially preserving responses to secondary infections, which are common and often deadly in influenza patients. H84T holds promise as a novel antiviral that combines control of viral replication with enhancement of the immune response.

Introduction

Influenza, caused by the influenza A virus (IAV), continues to cause significant mortality and morbidity worldwide. Seasonal influenza epidemics result in millions of infected individuals and cause 500,000 deaths annually. In addition, at irregular intervals the virus undergoes changes that can cause pandemics with high mortality [1]. Virus-specific immune responses are critical to control and clear the infection, but if not properly controlled, may also contribute to immunopathogenesis and development of more severe disease [2]. In addition, IAV has developed strategies to escape immunological control [3–8]. Influenza vaccines contribute significantly to reduced morbidity and mortality. However, vaccine effectiveness is impacted by the ever changing nature of the virus and influenza vaccines are known to be less effective among the elderly [9,10]. Thus, potent influenza-targeting antiviral agents would increase our ability to effectively prevent and/or treat influenza. Currently, there are only a few anti-influenza drugs in use clinically [11,12], and IAV strains resistant to these drugs are emerging worldwide [13–15]. Therefore, identification and development of novel anti-IAV therapies would be desirable to expand the toolbox available to combat influenza.

Lectins are a group of proteins characterized by their ability to recognize and bind to specific carbohydrates without modifying them [16]. Banana lectin (BanLec) in its native (wild type) form has broad antiviral effects against many enveloped viruses including IAV [16]. BanLec restricts IAV replication *in vitro* [17] mainly via specific

binding to high mannose bearing glycoproteins present at high densities on the surface of influenza virions [18,19]. However, clinical applications of BanLec were long considered impossible due to the strong mitogenic properties of the lectin with potential systemic inflammatory side effects [20,21]. A single amino acid substitution at position 84 of wild type BanLec (WT) from histidine to threonine (H84T), was shown to uncouple the mitogenicity from the antiviral activity of this lectin [12,17]. By binding to hemagglutinin (HA) and inhibiting virus-endosome fusion, genetically engineered H84T restricts replication across IAV strains, including drug-resistant strains, in mammalian cells [12]. *In vivo*, H84T protects mice from lethal IAV infection without causing mitogenic effects [12], indicating that H84T could potentially be applied as a broad-spectrum anti-influenza agent. However, to evaluate the clinical usefulness of H84T it is critical to determine the yet unstudied impact of the lectin on human immune cells and their function during viral infection. Dendritic cells (DCs) are innate immune cells that are well equipped to sense incoming viruses. DCs line the respiratory mucosa and are also recruited there in response to IAV infection [22]. Importantly, DCs are antigen-presenting cells with the unique capacity to activate naïve T cells and are therefore critical for initiating the expansion and activation of antigen-specific T cells that are necessary to control and clear infection [5,22]. We have previously shown that IAV infection impairs the capacity of DCs to present antigen to T cells, providing a partial explanation to why IAV infection is not only disease-driving on its own but also increases the risk of the secondary bacterial infections commonly seen in IAV patients [5]. Here we set out to determine the effects of BanLec on human DCs during IAV infection, including their ability to present viral antigen to and activate T cells, to evaluate the future use of H84T as an antiviral treatment in humans.

Results

WT BanLec, but not H84T, induces human T cell proliferation via dendritic cells in an HLA-DR- and CD86-dependent manner

We first confirmed that, as previously shown [17], H84T, unlike WT BanLec (WT), did not induce significant CD4 or CD8 T cell proliferation in human peripheral blood mononuclear cells (PBMCs) (Fig 1A and 1B) as compared to untreated PBMCs or PBMCs treated with D133G BanLec (D133G), a control lectin engineered to lose both antiviral and mitogenic capacity (Fig 1A and 1B). In contrast, in new experiments using only purified T cells, CD4 and CD8 T cells did not proliferate in response to BanLec (Fig 1A and 1B). Thus, we hypothesized that antigen-presenting cell (APC) - T cell interactions, present in PBMCs, may be important in mediating the mitogenic effect of BanLec. To test this hypothesis, we co-cultured autologous monocyte-derived dendritic cells (DCs) and purified T cells and found that WT induced significant CD4 and CD8 T cell proliferation compared to D133G or no BanLec (Fig 1C–1E), restoring the pattern observed in PBMCs. Again, H84T did not induce T cell proliferation (Fig 1C–1E), confirming lack of mitogenicity. Furthermore, blocking HLA-DR or CD86 in the DC-T cell co-cultures significantly decreased CD4 T cell proliferation induced by WT compared to the isotype control (Fig 1F), while only CD86 blocking significantly reduced WT-induced CD8 T cell proliferation (Fig 1G). Transfer of BanLec-stimulated DC supernatants did not induce T cell proliferation (Fig 1H and 1I), showing that the mitogenic effect of WT was dependent on DC-T cell contact. In summary, our data show that the mitogenic effect of WT is cell contact dependent in an HLA-DR- and CD86-dependent manner, while H84T displays no mitogenic effect on neither human PBMCs nor purified T cells.

H84T treatment of DCs results in higher IAV-specific T cell expansion in response to replicating virus

To assess the effect of H84T on the induction of virus-induced T cell proliferation, we next co-cultured autologous DCs and T cells in the presence or absence of replicating IAV (Fig 2A). IAV induced both CD4 (Fig 2B) and CD8 (Fig 2C) T cell proliferation in the absence of BanLec. As expected, the D133G treated cells displayed similar T cell proliferation as in the condition without BanLec (Fig 2B and 2C). In contrast, WT induced high levels of CD4 (Fig 2B) and CD8 (Fig 2C) T cell proliferation regardless of whether IAV was present or not, in line with its mitogenic capacity. Interestingly, in the

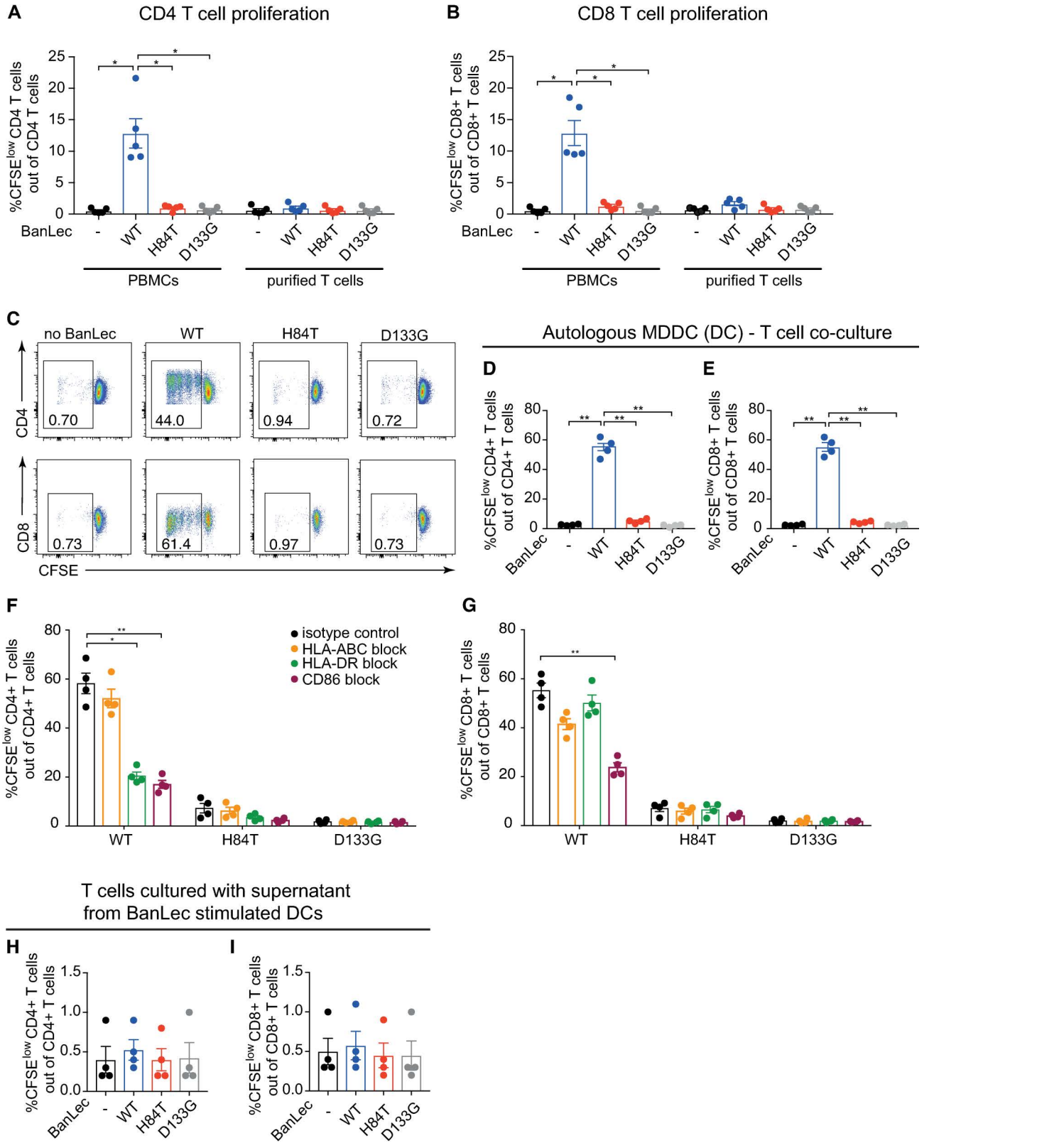

Fig 1. WT but not H84T, induces human T cell expansion by interacting with dendritic cells (DCs) in an HLA-DR and CD86-dependent manner. (A, B) CFSE labelled PBMCs or isolated T cells from the same donor were exposed to nothing (black) or to 2 μg/mL WT (blue), H84T (red) or D133G (grey) BanLec for 72 hours. T cell proliferation was detected by CFSE dilution using flow cytometry. Graphs show frequencies of live proliferating

CFSE^{low} **(A)** CD4 T cells and **(B)** CD8 T cells from five individual donors with mean ± SEM. **(C–E)** DCs were exposed to nothing or 2 μg/mL WT, H84T or D133G for 1 hour, and then co-cultured with CFSE labelled autologous CD4 or CD8 T cells (1:10 ratio MDDC:T cells) for 5 days. T cell proliferation was detected by CFSE dilution using flow cytometry. **(C)** Dot plots show live CD4 or CD8 T cells and numbers indicate frequency of proliferating CFSE^{low} T cells. One representative donor of four is shown. **(D, E)** Graphs show frequencies of live proliferating CFSE^{low} **(D)** CD4 T cells and **(E)** CD8 T cells in autologous DC-T cell co-culture from four individual donors. **(F, G)** DCs were treated with 3 μg/mL isotype control (black), anti-HLA-ABC (orange), anti-HLA-DR (green) or anti-CD86 (maroon) blocking antibodies for 1 hour, and then exposed to 2 μg/mL WT, H84T or D133G and co-cultured with CFSE labelled autologous CD4 or CD8 T cells (1:10 ratio DC:T cells) for 5 days. Bar graphs show frequency of live proliferating CFSE^{low} **(F)** CD4 T cells and **(G)** CD8 T cells from four individual donors with mean ± SEM. (**H-I**) DCs were exposed to nothing or 2 μg/mL WT, H84T or D133G and cultured for 24 hours, then the supernatants were collected and added to CFSE labelled autologous CD4 or CD8 T cells and cultured for 3 days. Bar graphs show frequency of live proliferating CFSE^{low} **(H)** CD4 T cells and **(I)** CD8 T cells from four individual donors with mean ± SEM. Friedman test with Dunn's multiple comparisons test was used to assess statistically significant differences at * $p < 0.05$ (** $p < 0.01$).

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presence of H84T, both CD4 (Fig 2B) and CD8 (Fig 2C) T cell proliferation was consistently significantly higher than in the IAV exposed co-cultures without BanLec or with D133G (Fig 2B and 2C). To estimate the level of T cell proliferation in response to IAV not associated with the mitogenic effect of BanLec, we compared the fold change CD4 and CD8 T cell proliferation by normalizing (dividing) proliferation in the no virus conditions across groups (Fig 2D and 2E). We found that the fold change IAV-induced CD4 and CD8 T cell proliferation was actually lowest in the WT condition (Fig 2D and 2E), which implies that the robust T cell expansion in the presence of WT was not antigen-specific. Interestingly, in the presence of H84T, the fold change of IAV induced CD4 and CD8 T cell proliferation was significantly higher compared to no BanLec or D133G treated cells (Fig 2D and 2E), suggesting that H84T does not impair DC antigen-presentation but in fact enhances T cell proliferation in response to replicating IAV.

To study the effect of H84T on IAV-specific T cell responses, we generated DCs and isolated CD8 T cells from HLA-A2 + donors with detectable IAV M1-specific CD8 T memory cells to monitor proliferation of IAV-specific CD8 T cells in the presence of BanLec and/or IAV (Fig 2A). As expected, IAV M1-specific CD8 T cells proliferated (upper left quadrant) in response to IAV in cultures without BanLec or with D133G, while no IAV-specific CD8 T cell proliferation was observed in the absence of IAV (Fig 2F and 2G). In cultures with WT, no or little IAV M1-specific CD8 T cell expansion was observed in response to IAV (Fig 2F and 2G). Again, this supports the conclusion that the substantial T cell proliferation induced by the WT is mitogenic rather than virus-specific. Interestingly, we again observed that exposing DCs to H84T and IAV resulted in higher frequencies of proliferating IAV-specific CD8 T cells compared to co-cultures with no BanLec or D133G (Fig 2F and 2G). Together, this shows that the modified plant lectin H84T promotes superior virus-specific T cell responses to replicating IAV.

H84T limits IAV infection in DCs, which preserves DC viability and allows expression of viral antigen for presentation to T cells

To understand why H84T treatment of IAV infected DCs results in generation of higher frequencies of virus-specific T cells, we studied DC-IAV-H84T interactions. We exposed DCs to replicating IAV in the presence or absence of BanLec and determined the viral infection by intracellular IAV nucleoprotein (NP) staining. The frequency of IAV NP + DCs was significantly reduced in the presence of H84T and WT as compared to no BanLec or D133G treated groups (Fig 3A and 3B). Importantly, H84T also efficiently protected also human primary blood and tonsil myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) from IAV infection (S1 Fig).

In response to pathogens including viruses, DCs undergo maturation characterized by upregulation of HLA-ABC (MHC I) and HLA-DR (MHC II), co-stimulatory molecules CD86/CD80 and CD40 to facilitate antigen-presentation and T cell interaction [23,24]. Therefore, we next tested the impact of H84T on the MHC and co-stimulatory molecule expression of DCs during IAV infection. As expected, IAV itself was a potent inducer of DC maturation, while the different BanLec variants did not induce DC maturation on their own (S2A–S2D Fig). In line with their ability to inhibit IAV infection, the presence of WT or

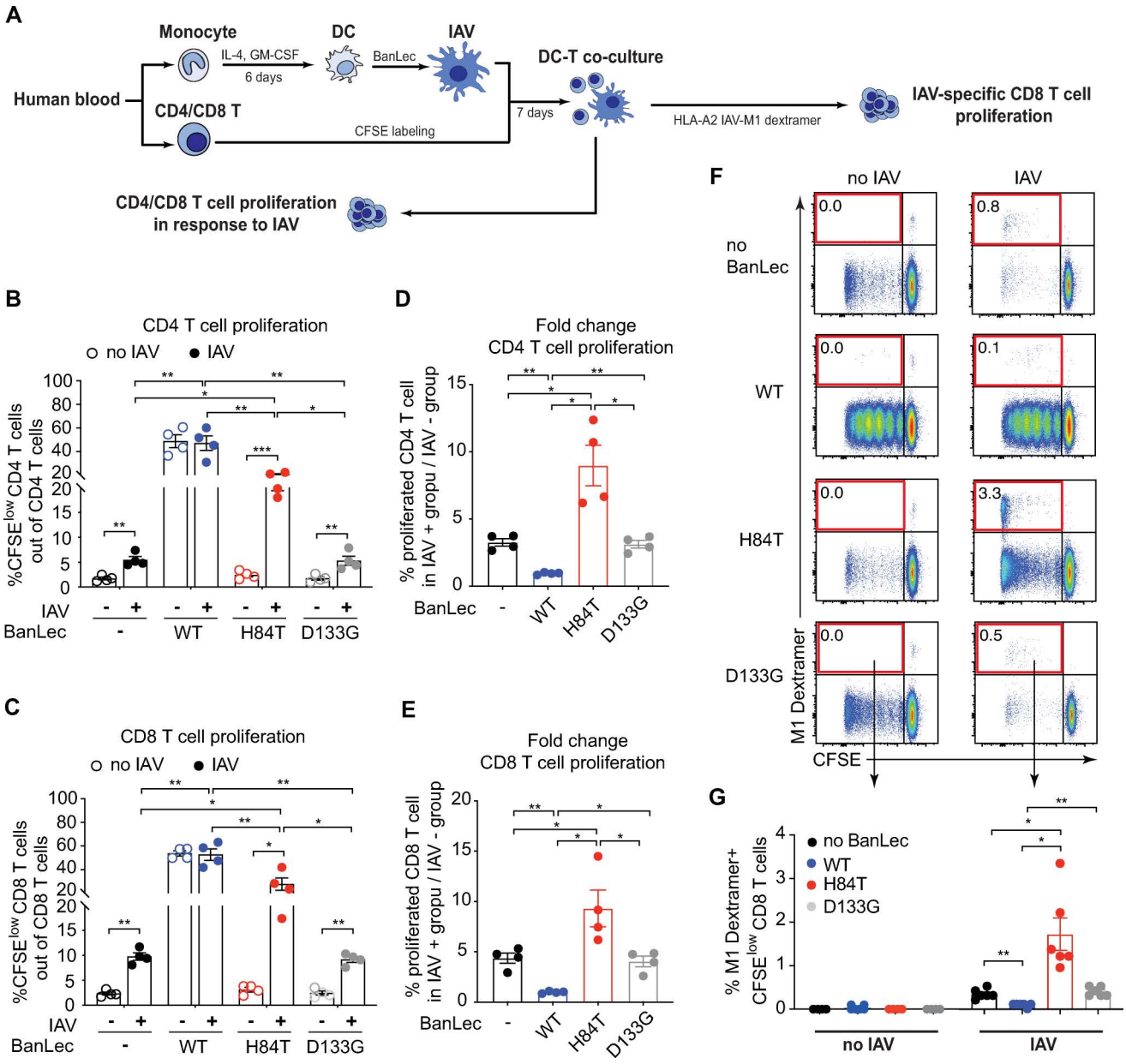

Fig 2. H84T treatment of DCs results in higher frequencies of IAV-specific CD8 T cells in response to replicating IAV. (A) DCs were exposed to nothing or 2 μg/mL WT, H84T and D133G for 1 hour and then exposed to no virus or 0.6 MOI IAV for 4 hours. Subsequently, CFSE labelled autologous CD4 or CD8 T cells were added (1:10 ratio MDDC:T cells) and co-cultured for 7 days. T cell proliferation was detected by CFSE dilution using flow cytometry. **(B, C)** Bar graphs show frequency of live proliferating $CFSE^{low}$ **(B)** CD4 T cells and **(C)** CD8 T cells from four individual donors with mean ± SEM. **(D, E)** Bar graphs show the fold change of proliferated **(D)** CD4 T cells and **(E)** CD8 T cells with mean ± SEM, in which the frequencies of IAV induced CD4 and CD8 T cell proliferation were divided by no virus conditions. **(F, G)** DCs from HLA-A2 positive donors with detectable IAV-specific CD8 T cell memory responses were generated and exposed to nothing or to 2 μg/mL WT, H84T or D133G for 1 hour, and then exposed to no IAV or 0.6 MOI IAV for 4 hours. Subsequently, CFSE labelled autologous CD8 T cells were added (1:10 ratio DC:T cells) and co-cultured for 7 days. IAV M1-specific CD8 T cells were identified with an HLA-A2 Influenza A M1 dextramer and analysed using flow cytometry. **(F)** Dot plots show live CD8 T cells from one representative donor. The $CFSE^{low}$ M1 $Dextramer^{+}$ population (upper left quarter of the plots) represents proliferated IAV-specific CD8 T cells detected by IAV M1 Dextramer. Frequencies of $CFSE^{low}$ M1 $Dextramer^{+}$ CD8 T cells out of total CD8 T cells are displayed. **(G)** Bar graphs show frequency of live $CFSE^{low}$ M1 $Dextramer^{+}$ CD8 T cells from six individual donors with mean ± SEM. Statistical differences were assessed using RM one-way ANOVA with Tukey's multiple comparisons test and considered significant at * $p < 0.05$ (** $p < 0.01$).

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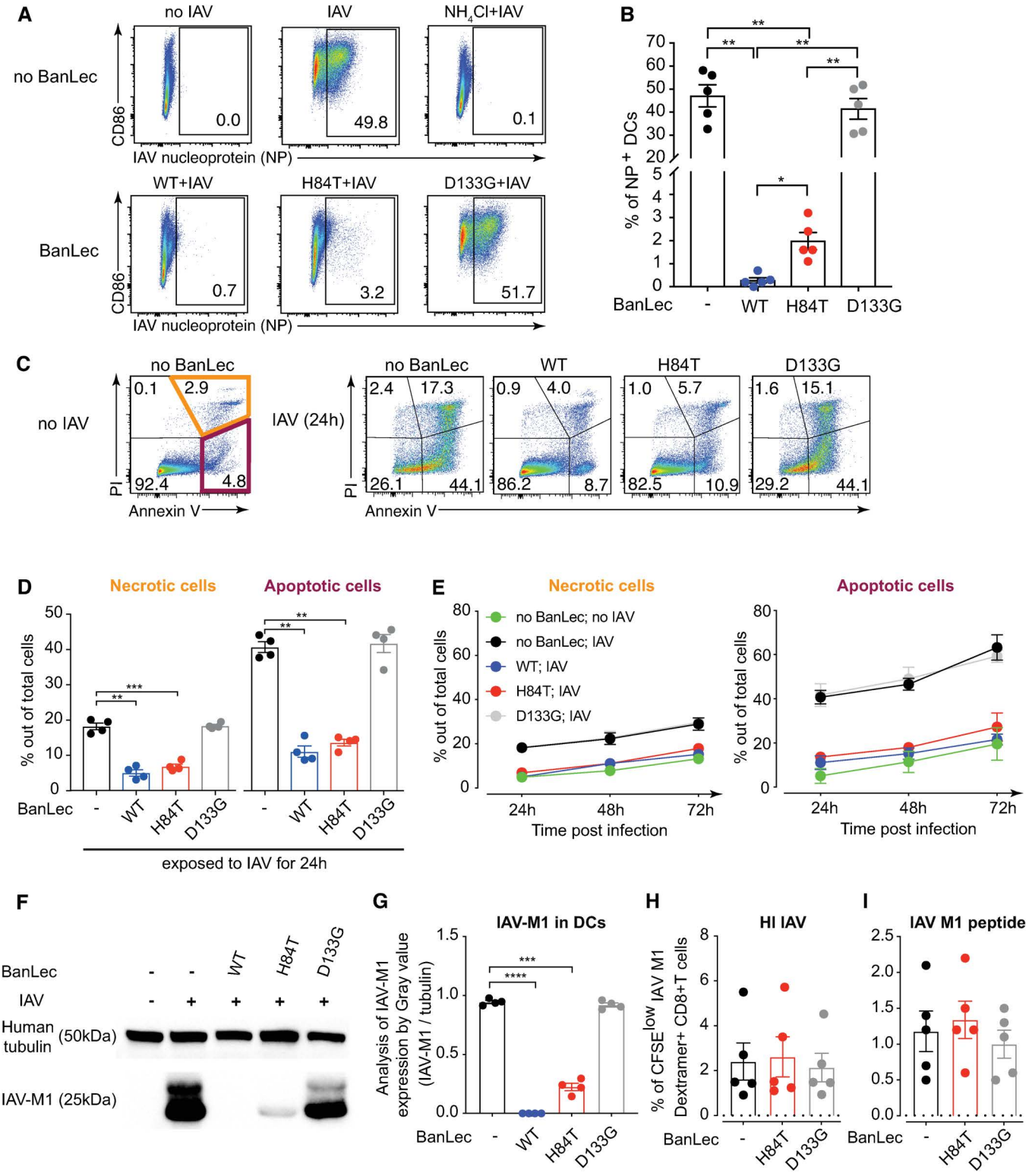

Fig 3. H84T effectively limits IAV infection in human DCs, and consequently preserves DC viability but allows expression of viral antigen for presentation to T cells. (A, B) DCs were exposed to nothing or 2 μg/mL WT, H84T or D133G for 1 hour, and then exposed to no IAV, or 0.6 MOI IAV with or without 20 mM NH_4Cl for 24 hours to prevent viral fusion. **(A)** Dot plots show live DCs and numbers depict the frequency of IAV nucleoprotein

(NP)+ DCs from one representative donor. **(B)** Graphs show frequency of IAV NP + DCs from five individual donors with mean ± SEM. **(C–E)** DCs were exposed to nothing or 2 μg/mL WT, H84T or D133G for 1 hour, then exposed to no IAV or 0.6 MOI IAV for 24, 48 or 72 hours. Viability of DCs was detected by Annexin V and PI staining using flow cytometry. **(C)** Dot plots show DCs and numbers depict the frequency of PI or Annexin V positive DCs from one representative donor out of four. **(D)** Graphs show frequency of necrotic (orange) and apoptotic (maroon) cells out of total cells after 24 hours of exposure to IAV in four donors with mean ± SEM. Paired t test was used to analyze the data and data considered significant at * $p < 0.05$ (** $p < 0.01$, *** $p < 0.001$). **(E)** Line graphs show the frequency of necrotic and apoptotic cells with mean ± SEM in four individuals. **(F, G)** IAV M1 protein in cell lysates from DCs exposed to nothing, 2ug/mL BanLec (WT, H84T or D133G) with or without 0.6 MOI IAV for 24 hours were determined by Western blot. Tubulin was used as the loading control. **(F)** One representative donor from four is shown. An overexposed blot is shown to highlight that, unlike in the BanLec WT treated cells, there is indeed a detectable band in H84T treated cells corresponding to IAV M1 protein. **(G)** Graph shows the analysis of IAV-M1 expression level by gray value in DCs exposed to replicating IAV using blots that were not overexposed. (**H, I**) DCs from HLA-A2 positive donors with detectable IAV-specific CD8 T cell memory responses were generated and exposed to nothing or to 2 μg/mL H84T or D133G BanLec for 1 hour, and then exposed to heat-inactivated IAV (HI IAV) or IAV M1 peptide (GILGFVFTL) for 4 hours. Subsequently, CFSE labelled autologous CD8 T cells were added and co-cultured for 7 days. IAV M1-specific CD8 T cells were identified with an HLA-A2 Influenza A M1 dextramer and analysed using flow cytometry. T cell proliferation was detected by CFSE dilution. Graph shows frequencies of live $CFSE^{low}$ M1 Dextramer+ CD8 T cells with **(H)** HI IAV and **(I)** IAV M1 peptide stimulation with mean ± SEM values from five individual donors. Dotted lines show the mean frequencies of live $CFSE^{low}$ M1 Dextramer+ CD8 T cells in DC-CD8 T cell co-culture without HI IAV or IAV M1 peptide stimulation. Statistical differences were assessed using the Friedman test with the Dunn's multiple comparisons test and considered significant at * $p < 0.05$.

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H84T resulted in less DC maturation as assessed by HLA-ABC, HLA-DR, CD86 and CD40 expression in response to IAV (S2A–S2D Fig). Similarly, replicating IAV induced secretion of TNFα and IL-6 from DCs but not in the presence of WT and H84T that inhibit infection (S2E and S2F Fig). Together, these findings show that greater DC maturation or higher cytokine secretion do not explain why H84T treatment results in superior propagation of virus-specific T cells.

We next determined the viability of IAV exposed DCs in the presence or absence of BanLec since IAV replication typically results in cytopathic effects, which in turn may affect the ability of DCs to interact with T cells. We used Annexin V and propidium iodide (PI) staining to identify apoptotic and necrotic cells respectively in the cultures after 24–72 hours of IAV exposure (Fig 3C–3E). After 24 hours of exposure to IAV, in the presence of both WT and H84T the frequencies of apoptotic and necrotic DCs were significantly lower compared to no BanLec (Fig 3C and 3D). WT and H84T treatment preserved DC viability 48h and 72h post infection (Fig 3E), showing that the antiviral property of both H84T and WT coincides with preserved viability of DCs in the presence of replicating IAV. This could also be observed when BanLec-treated DCs are exposed to circulating seasonal influenza virus strains including H1N1 (A/Michigan/45/2015) and H3N2 (A/Singapore/INFIMH-16–0019/2016) (S3 Fig), suggesting the broad-spectrum of antiviral property of both H84T and WT (S3A and S3B Fig), and efficiency in preserving viability of DCs during viral infection (S3C and S3D Fig).

Still, only H84T treatment, but not WT, resulted in higher frequencies of IAV-specific T cells. Therefore, we next determined viral antigen load in DCs under different culture conditions since that is another rate limiting step in antigen presentation and T cell activation. Cell lysates from IAV exposed DCs displayed a strong IAV M1 protein band when cultured without BanLec or with D133G (Fig 3F and 3G). In lysates of DCs exposed to H84T and IAV, a weak but visible M1 protein band was detected, while no M1 protein was detected in lysates of WT treated DCs (Fig 3F and 3G). These data show that H84T treatment, but not WT, allows limited but detectable viral antigen expression in IAV exposed DCs.

Taken together, H84T significantly limits IAV infection in DCs, which preserves DC viability but allows some viral antigen to be translated and available for T cell presentation. We propose that this explains why H84T treatment of DCs in the presence of replicating IAV results in higher frequencies of IAV-specific T cells as compared to untreated DCs (more cell death) or DCs treated with WT (no viral antigen and non-specific T cell proliferation). To test this hypothesis, we added non-replicating forms of IAV antigen to the DC-T cell co-cultures: heat-inactivated IAV (HI IAV) that can bind and fuse but not replicate, or IAV M1 peptide (GILGFVFTL). HI IAV (Fig 3H) and IAV M1 peptide (Fig 3I) could both induce similar IAV M1-specific CD8 T cell proliferation in co-cultures regardless of treatment (Fig 3H and 3I). Taken together, we found that H84T treatment improves virus-specific T cell responses in the presence of replicating IAV and, importantly, does not impair immune responses to non-replicating IAV antigens.

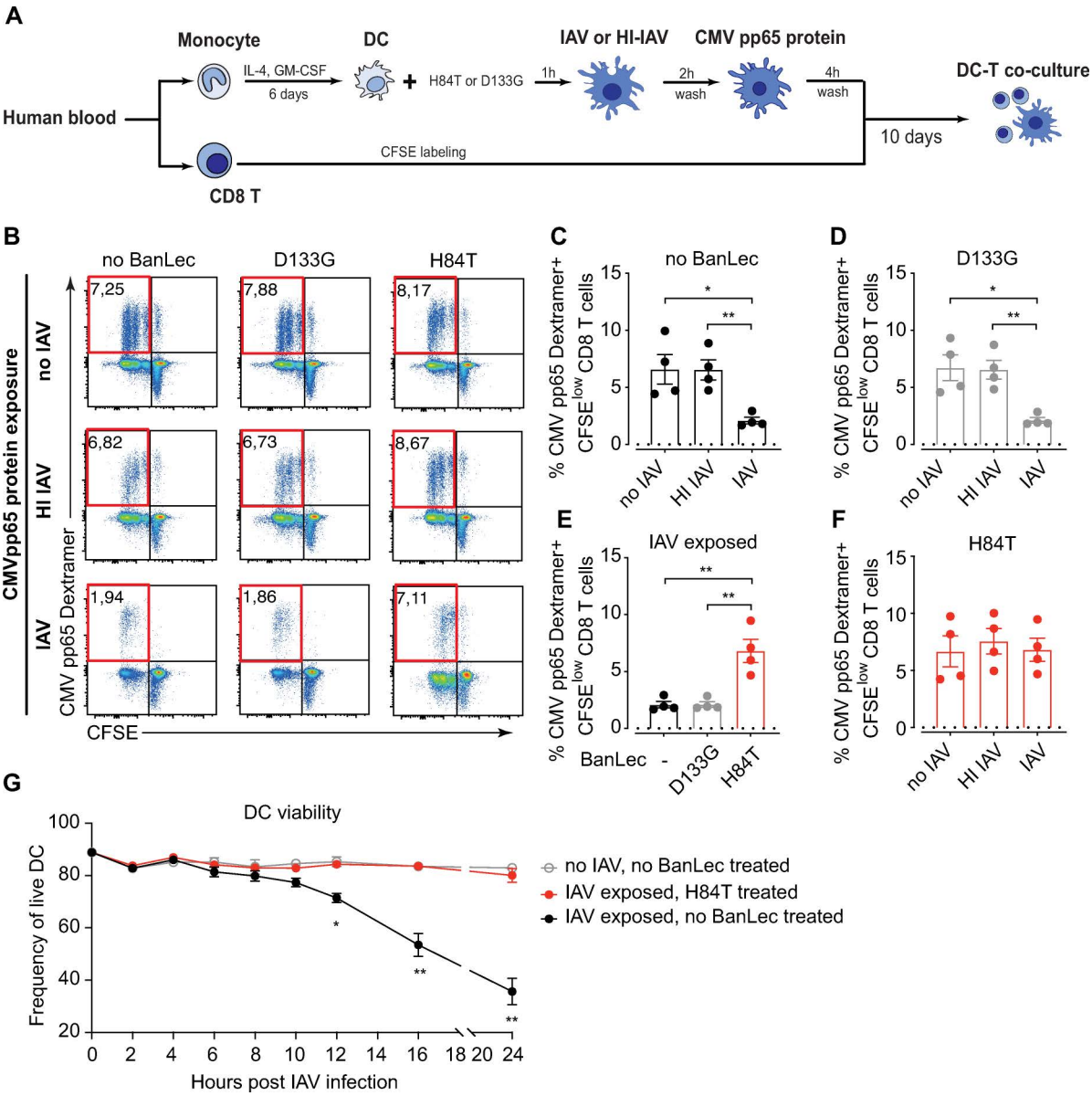

Fig 4. H84T restores the capacity of DCs to initiate pathogen-specific CD8 T cell expansion against CMV antigen post replicating IAV infection. (A) DCs differentiated from HLA-A2 + donors were pre-treated with nothing or H84T or D133G for 1h, and then exposed to IAV (0.6 MOI) or HI IAV (0.6 MOI) for 2h. Then the cells were washed and the recombinant cytomegalovirus (CMV) pp65 protein were added as a second source of antigen. After 4h incubation, DCs were washed and co-cultured with autologous CFSE labelled CD8 T cells for 10 days. CMV-specific CD8 T cells were identified with an HLA-A2-CMV pp65-dextramer and analysed using flow cytometry. **(B)** Dot plots show live CD8 T cells from one representative donor. The $CFSE^{low}$ CMV pp65 $Dextramer^{+}$ population (upper left quarter of the plots) represents proliferated CMV-specific CD8 T cells. Frequencies of $CFSE^{low}$ CMV pp65 $Dextramer^{+}$ CD8 T cells out of total CD8 T cells are displayed. Bar graphs show frequency of live $CFSE^{low}$ CMV pp65 $Dextramer^{+}$ CD8 T cells in conditions (**C**) without BanLec, or in the presence of **(D)** D133G or **(F)** H84T from four individual donors with mean ± SEM. **(E)** Bar graphs show frequency of live $CFSE^{low}$ CMV pp65 $Dextramer^{+}$ CD8 T cells in DC-CD8 T cell co-culture with replicating IAV exposure from four individual donors with mean ± SEM. **(C-F)** Dotted lines show the mean frequency of live $CFSE^{low}$ CMV pp65 $Dextramer^{+}$ CD8 T cells in DC-CD8 T cell co-culture without CMV pp65 protein stimulation. **(G)** DCs differentiated from HLA-A2 + donors were pre-treated with nothing or H84T for 1h, and then exposed to nothing or IAV (0.6 MOI) for 2h. Then the cells were washed and the recombinant CMV pp65 protein added as a second source of antigen. The viability of the DCs was detected by Annexin V and PI staining using flow cytometry at 0, 2, 4, 6, 8, 10,12,16 and 24h post IAV infection. Line graph shows the frequency of live DCs out of total DCs from four individual donors with mean ± SEM. Statistical differences were assessed using RM one-way ANOVA with Tukey's multiple comparisons test and considered significant at * $p < 0.05$ (** $p < 0.01$).

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H84T treatment of IAV infected DCs rescues their capacity to initiate specific CD8 T cell responses against other antigens

IAV infection predisposes patients to secondary bacterial infection, which increases the risk of severe or fatal disease [25,26]. We have previously reported that IAV infection of DCs impairs their ability to present antigen to CD8 T cells, which may contribute to increased susceptibility to secondary infections [5]. To assess the impact of H84T treatment of IAV exposed DCs on mounting T cell responses against non-IAV antigens, we used cells from HLA-A2 + donors with detectable CD8 T cell memory responses against CMV pp65 protein, which was added as a second antigen in autologous DC and CD8 T cell co-cultures exposed to replicating IAV or HI IAV in the absence or presence of BanLec (Fig 4A). CMV pp65-specific CD8 T cells did not proliferate in the absence of CMV pp65 protein (S4 Fig). As previously reported, DCs exposed to replicating IAV induced lower frequencies of CMV-specific CD8 T cells compared to DC not exposed to IAV or exposed to HI IAV (Fig 4B and 4C). As expected, D133G treatment generated similar results as did no BanLec (Fig 4B and 4D). Interestingly, H84T treatment resulted in significantly higher level of CMV-specific CD8 T cell proliferation by IAV infected DCs, as compared to IAV infected DCs exposed to D133G or not treated with BanLec (Fig 4B and 4E). In fact, in the presence of H84T, DCs exposed to replicating IAV induced similar CMV-specific CD8 T cell responses as did uninfected DCs or HI IAV exposed DCs (Fig 4B and 4F). Together, our data suggest that H84T rescues the impaired capacity of IAV infected DCs to induce CD8 T cell proliferation against other antigens, to comparable levels as seen in the absence of IAV infection.

To understand the mechanism of action behind this observation, we determined the viability of DCs longitudinally after exposure to replicating IAV or no IAV, in the presence of CMV pp65 protein. We found that, without BanLec treatment, the viability of DCs exposed to replicating IAV started to significantly decrease 12 hours post infection as compared to DCs not exposed to IAV (Fig 4G). In the presence of H84T, the viability of DCs was comparable to that in the conditions with no IAV (Fig 4G). Thus, H84T likely rescues antigen-presenting capacity of DCs exposed to replicating IAV by preserving DC viability.

To sum up, our data shows that H84T not only blocks IAV replication but also facilitates robust pathogen-specific CD8 T cell responses during IAV infection, which supports the continued development of H84T as novel antiviral against IAV.

Discussion

Since its identification in 1990 [27], BanLec has been studied as a potential broad-spectrum antiviral due to its strong binding affinity for high mannose found on many enveloped viruses [28]. However, systemic inflammatory responses in virus infection mouse models [29], driven by mitogenic activation and proliferation of T cells, has prevented the exploration of unmodified WT for clinical use. In contrast, the genetically engineered H84T is well tolerated in mouse models and does not induce T cell proliferation in human PBMCs [17]. H84T has potent antiviral effect and protects mice from lethal influenza infection, which supports continued development for therapeutic applications [12,17]. Still, the immunological mechanism of T cell proliferation, observed with WT but not with H84T, was unclear. Therefore, in this study, we first determined the impact of BanLec on human T cells. Interestingly, we found that WT did not induce proliferation by directly interacting with T cells. Instead, the mitogenic effect of WT required MHC II- and CD86-dependent APC-T cell interactions to induce T cell proliferation. This mechanism is similar to what has been described for superantigens such as staphylococcal enterotoxin A [30]. While T cells did not proliferate in the presence of H84T, even when co-cultured with DCs that are professional APCs. Thus, our data confirm the absence of mitogenic effect of genetically engineered H84T on human T cells.

In response to viral infections like influenza, activation and expansion of virus-specific T cells are critical for effective viral clearance and long-lived immunological memory. This requires well-orchestrated cellular interactions between T cells and APCs, especially DCs. Upon IAV infection, DCs sense and capture the virus for subsequent antigen processing and presentation [31–33]. Though airway epithelium is the initial host cell of influenza virus, studies have shown that airway DCs could be directly infected by influenza virus both in vivo and in vitro [34–36]. We have previously shown that IAV

infection impairs the capacity of DCs to present antigen to T cells, providing a partial explanation as to why IAV infection is not only pathogenic on its own but also increases the risk of the secondary bacterial infections commonly seen in IAV patients [5]. Thus, we determined the impact of H84T on the ability of DCs to support IAV-specific T cell expansion in response to replicating IAV. Interestingly, we found that H84T treatment resulted in more efficient expansion of IAV-specific CD8 T cells, despite the antiviral properties of H84T that limit viral replication and thus viral antigen availability. To investigate the mechanism of this observation, we determined DC maturation and cytokine production, necessary for T cell activation and expansion [37,38], in response to IAV infection during H84T treatment. We found that H84T did not induce superior DC maturation or cytokine production as compared to D133G or untreated cells. This indicates that H84T does not improve antigen-presentation of DCs directly. Instead, H84T might contribute to increased expansion of IAV-specific CD8 T cells via indirect factors.

IAV replication typically has cytopathic effects on infected cells and results in cell death in a dose- and time-dependent manner [39–44]. We found that both WT and H84T effectively limited viral replication, which resulted in preserved DC viability upon IAV exposure/infection. Importantly, H84T still allowed some viral protein production, providing sufficient antigen for DCs to present to and activate virus-specific CD8 T cells while limiting the cytopathic effects of IAV. This could explain why H84T but not WT treatment resulted in higher frequencies of proliferating IAV-specific T cells since WT prevented translation of any viral protein that could be presented to T cells. In addition, H84T treatment resulted in higher frequencies of proliferating IAV-specific CD8 T cells only in response to replicating IAV but not in response to non-replicating forms of the same antigen such as heat-inactivated IAV or IAV M1 peptide. In conclusion this suggests that H84T treatment efficiently limits viral replication resulting in maintained DC viability yet allowing some viral antigen to be available for presentation to virus-specific CD8 T cells. Thus, rather than actively promoting IAV-specific T cell proliferation in response to IAV infection, H84T sustains the capacity of DCs to initiate virus-specific T cell responses otherwise impaired by the cytotoxic effects of replicating IAV.

IAV infection of human primary DCs impairs their ability to present other antigens to CD8 T cells [5]. This may contribute to the increased susceptibility of influenza patients to secondary infections associated with more severe clinical outcomes [25,26]. We found the H84T could also restore the capacity of DCs to initiate robust pathogen-specific CD8 T cell expansion against CMV antigen during IAV infection. Here we used another viral antigen as our second antigen; in future studies it would be interesting to establish methods to assess if bacterial antigens are also more potently presented after H84T treatment, as bacterial infections are the typical secondary infection in influenza patients. By enabling the handling other pathogen-derived antigens in the presence of replicating IAV infection, our data indicate the H84T treatment may reduce the risk of severe disease in influenza.

In response to IAV infection, DCs produce inflammatory cytokines such as TNFα and IL-6 to initiate and modulate immune responses. However, too excessive cytokine production may contribute to cytokine storm, resulting in more severe disease [45]. Higher TNFα and IL-6 levels in influenza patients have been shown to correlate with severe disease or fatal outcome [46]. It is noticeable that H84T significantly decreased TNFα and IL-6 production of DCs induced by IAV without limiting virus-specific T cell expansion. Thus, our data also indicate the benefit of H84T treatment to reduce the risk of cytokine storm and progression of severe influenza.

Evaluation of H84T-specific T-cell or antibody responses is important for the future application of H84T BanLec as an anti-viral drug for human use. However, the experimental in vitro system we established and used in here is not suited to the study of the generation and dynamics of drug-specific T and B responses directed against H84T, as the cells are only kept in culture for 7 days. Likely, to evaluate drug-specific T cell or antibody responses, H84T BanLec needs to be administrated in vivo in humans, and adaptive immune responses to the lectin followed for periods of weeks or months. It has been previously reported that humans do have antibodies to BanLec and, as they are of the IgG 4 variety, they likely are tolerogenic [28]. Further, we have shown in mice, that administering H84T BanLec induces antibody production but that the anti-BanLec antibodies do not affect safety or the antiviral efficacy of H84T BanLec in vivo [12].

Developing an antiviral therapy that efficiently limits viral replication and cytopathic effects but allows the induction and expansion of virus-specific immune responses would be a valuable addition to existing influenza vaccines and therapies. In summary, our data support the future development of H84T as a therapeutic agent for use in humans to manage IAV infection, where its antiviral effects coupled with its ability to enhance virus-specific immune responses could be highly beneficial. In addition, H84T may also reduce the risk of severe disease by preventing excessive inflammatory cytokine production and enabling individuals to handle other pathogen-derived antigens after IAV infection. H84T also displays broad-spectrum antiviral effects in mouse and hamster models and cell lines on a wide range of enveloped viruses including influenza B virus (IBV), human immunodeficiency virus (HIV), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Middle East respiratory syndrome coronavirus (MERS), hepatitis C virus (HCV), Ebola virus and herpesviruses [12,17,47–51]. Future studies of the impact of H84T on human immune cell responses against those viruses would further justify the application of H84T as a broad-spectrum antiviral for use in humans.

Materials and methods

Ethics statement and human subjects

This study was approved by the Swedish Ethical Review Authority (Etikprövningsmyndigheten in Swedish) and performed according to the Declaration of Helsinki. Formal written consents were obtained from all study subjects in accordance with Ethical permits Dnr: 2011/1495-31/4 and 2017/1597-31. Human tonsils were obtained from adult patients undergoing routine tonsillectomies to treat obstructive sleep apnea. Informed consent was obtained from all donors. Buffy coats were obtained from healthy blood donors at the blood bank of Karolinska University Hospital.

BanLec

WT and genetically engineered H84T and D133G were prepared in E.coli and purified as previously described [17]. Briefly, cleared E.coli lysates were added to Ni-NTA agarose that has been equilibrated with IMAC-25 buffer. Then BanLec proteins were eluted via column and dialyzed against PBS using Slide-alyzer dialysis cassettes.

Isolation and culture of cells

MDDCs were differentiated and subsets of human primary DCs and T cells were isolated as previously described [5,52]. In brief, PBMCs were harvested after Ficoll-Paque Plus (GE Healthcare) gradient separation of buffy coats from healthy blood donors. Blood monocytes and T cells were enriched and obtained by counterflow centrifugal elutriation using RosetteSep human monocyte and T cell enrichment cocktail (StemCell Technologies), respectively. To differentiate MDDCs, monocytes were cultured with 40 ng/ml GM-CSF and 40 ng/ml IL-4 (both from R&D Systems) in complete medium, R10 (RPMI 1640 with 10% FCS, 1% L-glutamine and 1% penicillin/streptomycin) at 0.5×10^6 cells/ml. R10 was replaced on day 3 and immature MDDCs were harvested on day 6.

Blood and tonsil myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs) were obtained by using $CD1c^+$ Dendritic Cell Isolation Kit (Miltenyi Biotec) and the Diamond Plasmacytoid Dendritic Cell Isolation Kit II (Miltenyi Biotec) respectively, as previously described [52]. Briefly, tonsils were mechanically disrupted and filtered to get a single-cell suspension. Tonsil mononuclear cells (TMCs) were harvested after Ficoll-Paque Plus (GE Healthcare) gradient separation of single-cell suspension. Then, TMCs or PBMCs were labelled with a mixture of biotin-conjugated antibodies against lineage markers, Fc receptors, myeloid or plasmacytoid markers. The non-target cells were first depleted and then the target cells were further isolated by using anti-biotin Micro Beads and magnetic columns.

IAV

Influenza A/X31 (derived from influenza A/Aichi/2/68; H3N2) was propagated in chicken eggs, purified and concentrated on sucrose gradients (Virapur). The 50% tissue culture-infective does for IAV was determined by infecting a light

monolayer of MDCKs in the presence of trypsin and monitoring the cytopathic effect. Virus was replication incompetent after heat-inactivation at 56°C for 0.5 hour. The seasonal influenza virus strains H1N1 (A/Michigan/45/2015) and H3N2 (A/Singapore/INFIMH-16–0019/2016) were provided by Davide Angeletti, University of Gothenburg.

DC blocking assay

DCs, labelled with 0.25 μM CFSE (ThermoFisher Scientific), were treated with isotype control (purified mouse IgG, Biolegend), purified anti-HLA-ABC, anti-HLA-DR or anti-CD86 (all from Biolegend) blocking antibodies for 1 hour, and then exposed to WT, H84T or D133G and then co-cultured with CFSE labelled autologous T cells for 72 hours. T cell proliferation was detected by CFSE dilution using flow cytometry (LSRFortessa, BD Biosciences).

BanLec pre-treatment and IAV infection of DCs

DCs were pre-treated with nothing or WT, H84T or D133G for 1h, and then exposed to IAV for 24h. IAV infection was monitored using an anti-nucleoprotein (NP) antibody (Abcam) and flow cytometry. To prevent IAV infection, 20 mM NH_4Cl was added to DCs before they were exposed to IAV.

DC phenotype and cytokine secretion

After IAV infection, DCs were harvested, washed with PBS and surface stained with antibodies against CD1a, CD11c, CD14, HLA-DR, CD40, CD86, and CCR7 (all from BD Biosciences). Then DCs were washed, fixed, and analysed by flow cytometry. Supernatants were harvested and cytokines were measured by ELISA (TNFα and IL-6, R&D Systems).

DC viability after IAV infection

DCs were pre-treated with nothing or WT, H84T and D133G for 1h, and then exposed to IAV. DCs were harvested after 24, 48 and 72h respectively, washed in ice-cold PBS twice and resuspended in 1X Annexin binding buffer, and stained with Annexin V and propidium iodide (PI) (ThermoFisher Scientific), and analysed by flow cytometry within 0.5h of processing.

DC presentation of IAV to CD8 T

DCs differentiated from HLA-A2 + donors were pre-treated with nothing or WT, H84T or D133G for 1h, and then exposed to IAV (0.6 MOI), HI-IAV (0.6 MOI) or IAV M1 peptide (GILGFVFTL, 0.5 μg/mL) for 24h, and then co-cultured with CFSE labelled autologous CD8 T cells. After 7 days, cells were harvested and stained with HLA-A2 Influenza M1 (GILGFVFTL) dextramer (Immudex) for 15 min at room temperature followed by labelling with antibodies against CD3, CD4, CD8, CD69, CD14, CD19 and CD56 (all BD Biosciences), fixation and analysis by flow cytometry.

DC presentation of CMV pp65 protein to CD8 T

DCs differentiated from HLA-A2 + donors were pre-treated with nothing or H84T or D133G for 1h, and then exposed to IAV (0.6 MOI) or HI-IAV (0.6 MOI) for 2h. Then the cells were washed and the recombinant cytomegalovirus (CMV) pp65 protein (ThermoFisher Scientific) was added as a second source of antigen. After a 4 h incubation, DCs were washed and co-cultured with autologous CFSE labelled CD8 T cells. After 10 days, cells were harvested and stained with HLA-A2-CMV pp65-dextramer (Immudex) for 15 min at room temperature followed by labelling with antibodies against CD3, CD4, CD8, CD69, CD14, CD19 and CD56 (all BD Biosciences), fixation and analysis by flow cytometry.

IAV antigen load in DCs

After pre-treatment with nothing or WT, H84T or D133G for 1h, followed by IAV exposure for 24h, DC were harvested and lysed in SDS lysis buffer (Sigma-Aldrich). DNA was sheared mechanically and lysates were snap frozen on dry ice.

Lysates were run on 4–12% Bis-Tris reducing gel, transferred to a PVDF membrane and blotted for viral proteins with the anti-IAV M1 monoclonal antibody (Abcam). Human tubulin was used as a loading control.

Statistical analysis

Data were analysed using Prism 9 (GraphPad). Grouped data are generally presented as mean ± SEM. Comparisons between variables were performed using the Friedman test with Dunn's multiple comparisons test or paired t test where appropriate. A significance level of 95% was used and all tests were two-tailed.

Supporting information

S1 Fig. WT and H84T inhibit IAV infection of human primary myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs) from both blood and tonsils. pDCs and CD1c + mDCs were isolated from human blood and tonsil. DCs were exposed to nothing or 2 μg/mL WT or H84T for 1 hour, and then exposed to no virus or 0.6 MOI IAV for 24 hours. (A) Dot plots show live blood mDCs and numbers depict the frequency of IAV NP + mDCs from one representative donor out of three. (B) Dot plots show live blood pDCs and numbers depict the frequency of IAV NP + pDCs from one representative donor out of three. (C) Dot plots show live tonsil mDCs and numbers depict the frequency of IAV NP + mDCs from one representative donor out of two. (D) Dot plots show live tonsil pDCs and numbers depict the frequency of IAV NP + pDCs from one representative donor out of two. Graphs show frequency of IAV NP+ (E) blood mDCs, (F) blood pDCs, (G) tonsil mDCs and (H) tonsil pDCs with mean ± SEM. (E, F) Paired t test was used to assess statistically significant differences at * $p < 0.05$ (** $p < 0.01$).
(TIF)

S2 Fig. WT and H84T prevent IAV-induced maturation of DCs. DCs were exposed to nothing or 2 μg/mL WT, H84T or D133G for 1 hour and then exposed to no virus or 0.6 MOI IAV for 24 hours. The surface expression of (A) HLA-ABC, (B) HLA-DR, (C) CD86 and (D) CD40 on DCs in the absence or presence of different types of BanLec without IAV after 24 hours was measured by flow cytometry and displayed as geometric mean fluorescence intensity (MFI) from five individual donors with mean ± SEM. Supernatants were collected and the concentration of TNF and IL-6 was determined by ELISA. Graphs show the concentration of (E) TNF and (F) IL-6 from five donors with mean ± SEM. RM one-way ANOVA with Tukey's multiple comparisons test was used to assess statistically significant differences at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.
(TIF)

S3 Fig. H84T effectively inhibits multiple IAV strains to infect human DCs, and consequently preserves DC viability. DCs were exposed to nothing or 2 μg/mL WT or H84T for 1 hour, then exposed to no virus or 0.6 MOI IAV for 24 hours, including H1N1 (A/Michigan/45/2015) and H3N2 (A/Singapore/INFIMH-16–0019/2016). (A) Dot plots show IAV nucleoprotein (NP) and CD86 expression in live DCs and numbers depict the frequency of NP + DCs. (B) Graphs show frequency of IAV NP + DCs from three individual donors with mean ± SEM. (C, D) Viability of DCs was detected by Annexin V and PI staining using flow cytometry. (C) Dot plots show DCs and numbers depict the frequency of PI and/or Annexin V positive DCs from one representative donor out of three. PI and Annexin V double negative population are live. (D) Graphs show frequency of live DCs from three individual donors with mean ± SEM. Paired t test was used to assess statistically significant differences at * $p < 0.05$ (** $p < 0.01$).
(TIF)

S4 Fig. Frequency of CMV-specific CD8 T cell proliferation in autologous DC co-cultures without CMV pp65 protein stimulation. DCs differentiated from HLA-A2 + donors were pre-treated with nothing or H84T or D133G for 1h. Then the cells were washed without IAV or CMV pp65 exposure, and co-cultured with autologous CFSE labelled CD8 T

cells for 10 days. CMV-specific CD8 T cells were identified with an HLA-A2-CMV pp65-dextramer and analysed using flow cytometry. (A) Dot plots show live CD8 T cells from one representative donor. The $CFSE^{low}$ CMV pp65 Dextramer+ population (upper left quarter of the plots) represents proliferated CMV-specific CD8 T cells. Frequencies of $CFSE^{low}$ CMV pp65 Dextramer+ CD8 T cells out of total CD8 T cells are displayed. (B) Bar graphs show frequency of live CFSElow CMV pp65 Dextramer+ CD8 T cells in conditions without BanLec, or in the presence of D133G or H84T from four individual donors with mean ± SEM. Statistical differences were assessed using RM one-way ANOVA with Tukey's multiple comparisons test and considered significant at * $p < 0.05$.
(TIF)

S1 Data. Raw data that underlies this paper.
(XLSX)

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