**Discussion**

**Network Analysis**

The final network is adequate to describe the 3 specified states and the generated rules allow transitions between them. The 64 filtered solutions of this network lead to 2 attractors, which are equally common. The only difference between 2 attractor states lies in activity of the protein complex NFATC1:JUN-FOS:IRF4:BATF. Out of 11 TF present in the GRN, besides BTLA and NFATC1:JUN-FOS:IRF4:BATF other nodes had been given a fix value in the Bonesis input table **(Reference to it)**. Therefore, the output of attractors for these nodes is a prediction for activity of these elements during exhaustion. Although primary Bonesis output analysis showed that all nodes have at least one conditional rule **(Reference to it)**., in the final 64 solutions NFATC1 has a constant 0 as its only rule **(Reference to it)**.

**B- and T-lymphocyte attenuator**

Role of B- and T-lymphocyte attenuator (BTLA) has been investigated in case of a chronic infection caused by a different virus, hepatitis B. Although overall expression of BTLA in patients with this condition and healthy individuals was not significant, this TF was still identified as inhibiting depending on the stage of the viral infection (Yu et al., 2019).

Further studies indicate that BTLA absence triggers uncontrolled inflammation and T-cell response (Oya et al., 2008). However, others attribute the exact same fact to reduction of effector and memory T-cells (Flynn et al., 2013).

BTLA was found to coactively with Grb-2 promote PI3K, which encourages T-cell activation. For instance, this association happens also via CTLA-4, but not PD-1 (Gavrieli and Murphy, 2006).This highlights the difference in these 3 TCR inhibiting receptors. In Bolouri et al network PI3K activates AKT, which in its turn activates, BACH2, NFATC2, NFKB1 and AP1 and inhibiting FOXO1. In the cells analyzed during this work, out of activated TFs all but NFATC2 are more active or expressed in memory state; FOXO1 expression was established as exhausted signature in the cells. Hence, BTLA indirectly activates memory state TFs and inhibits an exhausted state TF.

The ligand of BTLA, HVEM, has been shown to act as a “molecular switch” depending on its ligating partner. Interaction with LIGHT activates immune response. Nonetheless, when it binds to BTLA it can provide both activation and inhibition. Once operating together, BTLA reduces cytokine production and interactions with TCRs, meantime HVEM activates NFKB1, as in case of HVEM-LIGHT interaction (Shui et al., 2011). Another revelation is that effects of HVEM-BTLA dependent whether interaction is cis or trans. Cis interaction inhibits the trans and is considered overall inhibitory. Trans interaction is the one that activates the NFKB1 pathway (Cheung et al., 2009). These facts advocate for a possible positive feedback mechanism between BTLA and NFKB1, that is yet to be considered in this network.

Numerous pieces of evidence hint BTLA action being both favorable and destructive for CD8+ T-Cells. The GRN points out that in all 64 solutions BTLA is eventually downregulated in the exhausted state. Therefore, this analysis suggests that BTLA interaction with TCRs is less detrimental for the exhaustion and that this inhibition is more reliant on PD-1 and CTLA-4 upregulation in this state. After all, the above-mentioned studies also indicate the difference between these 3 inhibitory TFs.

Finally, HVEM, also known as CD270 or TNFRSF14, should be considered as a new substantial element during T-cell exhaustion and in general antiviral response. As well as BTLA, it has been shown to bidirectionally influence T-cells. Interestingly, it has not been identified neither in Bolouri et al. network, nor in interactions derived from pySCENIC.

**NFATC1:JUN-FOS:IRF4:BATF**

NFATC1 and JUN-FOS act together to enforce a pro-memory state in the T-cells. However, acting alone NFATC1 promotes exhaustion. The same applies to BATF and IRF4. Together they an act to reduce exhaustion, which was shown in framework with CAR T-cells (Seo et al., 2021). Despite that finding, these two TFs were also classified as exhaustion driving by activating receptors like PD-1, which is a well-known exhaustion marker. (Man et al., 2017). Besides that, other study implies feedback mechanism, where PD-1 upregulates BATF (Quigley et al., 2009). NFATC1 as part of both resulting and the Bolouri et al. networks also activates PD-1 and CTLA4 receptors. Interestingly, it does not activate BTLA, which coming back to the previous point once again emphasizes the difference between nodes, that appear so similar in the context of the created network.

Remarkably, JUN-FOS is the only TF out of the 4 present in the protein complex that inhibits its activity. It is also present in the end GRN. This hints that JUN-FOS may be the TF that holds this complex under control, motion that is supported by the literature: JUN-FOS direction of action remains clearer, as it is considered the TF that prevents others from inducing exhaustion. The predominant idea behind these interactions is that the lack of balance between activities of these TFs and not one of them in particular is what drives the exhaustion, which reaffirms the statement for this protein complex the molecular context is everything (Papavassiliou and Musti, 2020).

In the Bolouri et al. network these 4 genes were acting cooperatively. As already explained, GRN in this project excludes such type of interactions and therefore they are considered as one entity. This kind of simplification removed direct interactions, like NFATC1 to JUN-FOS, but provided a new insight on how the complex interacts with the rest of the system. Other interacting partners from Bolouri et al. network were not included in the final network, IRF4 and BATF were discarded during filtering. They potentially could have brought more clarity in the functioning of the protein complex, since they form part of it.

Still no concrete remarks can be driven concerning cooperation of these TFs neither from the literature, nor from the GRN. Available expression data of these 4 genes separately excludes the possibility of assigning the complex to one state **(Reference to it)**.The two attractor states are split exactly in 1:1 proportion, when it comes to the activity of this protein complex. This suggests that, under current network architecture, the protein complex is less informative in describing CD8+ T cell exhaustion, when compared to other nodes.

**Nuclear factor of activated T-cells cytoplasmic 1**

Interactions with nuclear factor of activated T-cells cytoplasmic 1 (NFATC1) result insufficient to determine logical rules, that drive its switch from active in memory state to in active in exhausted, according to scRNA-seq data. In the Bolouri et al. network there are only two incoming interactions to this TF and both have been included. Although NFATC1 made it through the RF filtering, none of its interactions have done it, which is the reason why it is no additional input to explain its state could have come from the pySCENIC part of the network. RF importance score has been a great criteria to reduce the number of TFs to be considered. However, in this case it might have been too strict and created this obstacle, where it is impossible to increase complexity of NFATC1, without abandoning the consistency of the selection process.

After reanalyzing all the NFATC1 interactions that made the frequency filtering, but were discarded at the RF stage. one interaction could have potentially been used to solve the issue with the node. ETV3 has outgoing interactions to NFATC1 (48 pySCENIC runs) and NFKB1 (50 pySCENIC runs) and an incoming from JUND (50 pySCENIC runs) and NFKB1 (50 pySCENIC runs). According to ENCODE database, NFATC1 is for a fact a target of ETV3. Another database, JASPAR, confirms interaction from NFKB1 to ETV3. Moreover, a coexpression between NFKB1 and ETV3 has been reported in context of human monocytes and macrophages with regard to histone deacetylases (Ghiboub et al., 2020). All 3 possible interaction partners of ETV3 are interacting network. However, since it was dropped at the RF step of filtering, ETV3 was not considered significant enough to predict the cell states. Besides that, introducing a feedback loop with NFKB1 and two more interactions would most like excessively elevate the amount of solution networks.

**Network architecture**

The most connected node in this network is the one representing TCRs, it possesses 3 incoming, inhibiting links and 1 outgoing activating interaction. The inhibition inputs PD-1 and CTLA-4 are controlled together by NFAT family and are both on in the attractor state. Contrastingly, BTLA is regulated by different TFs than these two and is off in the attractor, which allows TCRs to stay on in the exhausted state. It has already been stressed out that even though BTLA has the same kind of interaction with TCRs as PD-1 and CTLA4, it appears to have a different influence on CD8+ T-Cell exhaustion, manifesting as bidirectional activity (Shui *et al.*, 2011). Considering these facts, the network can be split into two feedback loops, where protein complex NFATC1:JUN-FOS:IRF4:BATF links them together and EZH2 plays a role in both **(make a figure)**.

**Comparison with Bolouri Network**

**Transcription factor assignment**

Although the resulting network heavily relies on Bolouri et al. network, it extrapolates interactions and their sign from the reference network on the available scRNA-seq data. This empirical data led us to distinctly classifying 3 nodes from Bolouri et al. network.

In terms of activity, EZH2, NFATC1 and NFATC2 were assigned to a different cell state. Going back to the state of assigning the distinctive cell type to each TF, in case of EZH2 the selection was straightforward, as it is undoubtably more active in exhausted cell state. In EZH2 the difference between most active and second most active state is 21%. For NFATC2 the activity difference between top two state is smaller 16%. Meantime for NFATC1 its 11%. Considering this, it may be speculated that in repeated AUC score binarization NFATC1 would be the TF with highest likelihood to change bits most active cell state.

Moving to the biological reasons of these misalignments between final and reference networks, the action controversy of NFATC1, has been already covered earlier on. Similarly in case of NFATC2, whether NFATC2 enforces exhaustion or memory state strongly depends on the availability of its main interacting partner, JUN-FOS (Martinez et al., 2015). In the final network both members of the NFAT family activate exhaustive receptors, showing a controversy of its own, since NFATC1 was assigned to memory cell state **(Heatmap)**. Evidentially, there is no solid conclusion about these TFs and their action field seems to be closely intertwined and reliant on other factors, such as the isoform of a protein (Macian, 2005). EZH2 has been determined to have bidirectional effect in T-cells, while Bolouri et al. network showed that the timing of the EZH2 action makes it a more memory-like TF, there is also evidence supporting the contrary idea. In cancer context signature of terminally exhausted tumor-infiltrating lymphocytes strongly resembles CD8+ T-cell exhaustion signature during chronic viral infection (Miller et al., 2019). High expression of EZH2 has been reported to be a factor reducing survival of patients with hepatocellular carcinoma (Wu et al., 2022). Furthermore, its inhibition has been a sign of improvement in effectivity of immune checkpoint blockade therapy (Goswami et al., 2018).

Bolouri et al. network did not classify any of the TCRs and left them as another category. In case of this GRN all nodes have been given a definite cell state at some point (considering activity, gene expression or attractor states). PD-1 and CTLA-4 have been correctly interpreted as exhaustion markers using the dot plot **(Dotplot)**. BTLA assignment has been already discussed in detail and looks persuasive. The upregulation of CD28 and other TCRs in exhausted state seems contraintuitive and is yet to be properly reported. However, the dot plot showed it being upregulated in the exhausted state and downregulated in the transient state, possibly hinting a new mechanism within exhaustion. Bringing it all together, Bonesis has delivered feasible solutions with the selected interpretation of ambiguous TFs, demonstrating that there is still a room for interpretation concerning their roles. Concluding, these misalignments with Bolouri et al. network have a place to be, since they are supported by experimental data, literature and the GRN.

**New interactions**

An important feature of the resulting network are the 4 interactions added from pySCENIC, 2 of which were confirmed by Cistrome: JUND to NFKB1 (49 pySCENIC runs) and EZH2 to JUN (50 pySCENIC runs).

The STAT1 interactions have not been confirmed by sources implemented in this project. EZH2 interacting with STAT1 (50 pySCENIC runs) has indeed been reported, however not as a promotion but as an inhibition (Wee et al., 2014). In fact, STAT1 pathway is essential for the memory cell development. However, the setup of that study was investigating STAT1 effects in a vaccine setup, which resembles more acute, rather than chronic viral infection (Quigley et al., 2008). The interaction from STAT1 to NFATC2 (50 pySCENIC runs) has not been documented in CD8+ T-cells and only seems to have a less significant and indirect effect on this protein in CD4+ T-cells (Diehl et al., 2002). ENCODE database identifies this interaction as possible.

Ultimately, JUN-FOS interactions from pySCENIC seem more viable than the ones with STAT1.Nonetheless, exhausted cells in this study show an undoubtable upregulation and enhanced activity of STAT1, which may be another characteristic component of the analyzed cells.

**Outlook and Conclusion**

This GRN has the potential to be used fir texting in silico knock outs or upregulations. For example, by changing cell state characteristics (Figure XXX is an example of such) in memory or transient state the exhausted attractor state can be made unreachable. Thus, key exhaustion nodes and mutations can be identified. Other ways of simulating perturbation in the model, would be simulating treatment strategies and observing their effect on network dynamics. For instance, interferon therapy or check point blockade can be simulated and their consequences on STAT1, PD-1 and CTLA-4 can be examined.

From this study it has become even more evident that the interaction between NFATC1, JUN-FOS, IRF4 and BATF is meaningful during exhaustion. Nonetheless, the complexity behind this protein complex is as high as its importance. Hence, a separate network to study communication of these 4 genes would definitely bring more insights and possibilities for a breakthrough. This would also doubtlessly bring more clarity in the functioning of NFATC1, which is the weakest link of this network.

To summarize, the resulting GRN has demonstrated that empirically driven data can be successfully incorporated into already existing literature evidence and as the result provide a functioning model. This BN helps to identify focal points, where more knowledge is required, therefore becoming a backbone for a fuller model to describe CD8+ T-cell exhaustion. Advances analytical methods and an inevitable increase in data quality coming with it mark a bright future for such research.

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