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THE ROLE OF INTERFERON REGULATORY FACTOR 5 (IRF-5) IN CD4$^+$ T CELL DEATH DURING HIV INFECTION

By

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RÉSUMÉ

Le virus de l'immunodéficience humaine (VIH)/syndrome d'immunodéficience acquise (SIDA) reste un problème de santé publique mondial et un défi majeur dans le monde entier. Depuis le développement de la thérapie antirétrovirale (ART), des millions de personnes vivant avec le VIH ont pu contrôler la réplication virale, rétablir leur taux de CD4 et mener une vie normale. Cependant, malgré la thérapie, des niveaux résiduels d'inflammation, un faible degré persistant d'activation immunitaire et une plus grande sensibilité à l'apoptose cellulaire dans le compartiment des cellules T $CD4^+$ mémoires persistent chez la plupart des individus. Certains patients ne parviennent pas à récupérer leur taux de CD4. À ce jour, les mécanismes responsables de cette sensibilité élevée restent inconnus.

Dans notre quête pour comprendre pourquoi le maintien des cellules T $CD4^+$ dans les environnements inflammatoires chroniques est altéré, nous avons précédemment identifié le facteur de transcription Interferon Regulatory Factor 5 (IRF-5) comme étant un facteur de transcription crucial qui est responsable de la mort des cellules T $CD4^+$ lors de la leishmaniose viscérale expérimentale chronique (LV). Notre laboratoire a rapporté que l'activation de l`IRF-5 via le Toll-like receptor 7 (TLR7) par des fragments cellulaires apoptotiques dérivés de lésions tissulaires inflammatoires activait la caspase 8 et menait finalement à la mort cellulaire. Dans cette thèse, nous avons cherché à savoir si la voie TLR7/IRF5 était également activée au cours de l'infection par le VIH.

Nous avons montré que les cellules T $CD4^+$ mémoire régulent à la hausse TLR7 et IRF-5 chez les personnes infectées par le VIH (PLWH). En effet, l'IRF-5 était significativement régulé à la hausse dans les cellules T $CD4^+$ mémoire provenant de participants primo-infectés par le VIH-1 (PHI) et de PLWH sous traitement antirétroviral (ART), par rapport aux contrôleurs d'élite (EC) naturellement protégés et aux participants contrôles non-infectés par le VIH. En outre, l'expression de TLR7 a été maintenue après ART, et les expressions de IRF-5 et de TLR7 ont été directement corrélées avec AnnexinV et inversement corrélées avec le nombre de cellules T $CD4^+$ chez les PLWH. Lorsque nous avons examiné les partenaires de signalisation en amont et en aval d'IRF-5, nous avons constaté que TLR7 induisait IRF-5, favorisant l'expression de la Caspase 8 dans les cellules T $CD4^+$ des participants séropositifs pour le VIH-1 sous traitement antirétroviral, mais pas dans celles des participants non séropositifs. Il est intéressant de noter que l'activation de l'axe TLR7/IRF-5 prédisposait les cellules T $CD4^+$ à mémoire à l'apoptose

médiée par Fas en alimentant la voie Fas/FasL. Cette prédisposition a pu être bloquée à l'aide de peptides inhibiteurs de l'IRF-5.

Dans l'ensemble, nos résultats démontrent qu'en exprimant TLR7 et IRF-5, les cellules T $CD4^+$ mémoires conservent une sorte d'empreinte après le traitement antirétroviral qui les rend plus sensibles à l'apoptose médiée par la voie Fas/FasL.

Ce travail contribue à faire progresser nos connaissances sur les voies responsables de la perte des cellules mémoires chez les PLWH sous traitement antirétroviral et propose IRF-5 comme nouvelle cible thérapeutique pour prévenir la perte des cellules T $CD4^+$ mémoires chez les personnes séropositives.

Mots-clés : cellules T $CD4^+$, IRF5, VIH, apoptose.

ABSTRACT

The Human Immunodeficiency Virus (HIV)/Acquired Immune Deficiency Syndrome (AIDS) remains a significant global public health concern, persisting as a major challenge worldwide. Since the development of antiretroviral therapy (ART), millions of people living with HIV (PLWH) have been able to control viral replication, recover their CD4 count and lead a normal life; however, despite therapy, residual levels of inflammation, persistent low degree of immune activation, and higher sensitivity to cell apoptosis in the memory $CD4^+$ T cell compartment persist in most individuals and some patients do not achieve recovery of CD4 cell counts. To date, the mechanisms responsible for this high sensitivity remain elusive.

In our quest to understand defective maintenance of $CD4^+$ T cells in chronic inflammatory environments, we previously identified the transcription factor interferon regulatory factor 5 (IRF-5) as a crucial transcription factor that is responsible for $CD4^+$ T cells death during chronic experimental visceral leishmaniasis (VL). Our laboratory has reported that the activation of IRF-5 via Toll-like receptor 7 (TLR7) by inflammatory tissue damage-derived apoptotic cell material induced caspase 8 and ultimately led to cell death. In this study, we investigated whether the TLR7/IRF5 pathway is also activated during HIV infection.

We show that memory $CD4^+$ T cells upregulate TLR7 and IRF-5 in PLWH. Indeed, IRF-5 was significantly upregulated in memory $CD4^+$ T cells from primary HIV-1 infected participants (PHI) and from PLWH undergoing ART, when compared with naturally protected elite controllers (EC) and HIV^{free} control participants. Additionally, TLR7 expression was maintained after ART and IRF-5 and TLR7 expressions directly correlated with AnnexinV and inversely correlated with $CD4^+$ T cell counts in PHI. When we looked into upstream and downstream IRF-5 signalling partners, we found that TLR7 induced IRF-5, promoting caspase 8 expression in $CD4^+$ T cells from ART but not from HIV^{free} participants. Interestingly, activation of the TLR7/IRF-5 axis predisposed memory $CD4^+$ T cells to Fas-mediated apoptosis by feeding into the Fas/FasL pathway. This predisposition could be blocked using IRF-5 inhibitory peptides.

Taken together, our findings demonstrate that, by expressing TLR7 and IRF-5, memory $CD4^+$ T cells maintain a sort of imprint after ART that makes them more susceotible to Fas-mediated apoptosis.

This work contributes to advance our knowledge on the pathways responsible for memory cell loss in PLWH on antiretroviral therapy and proposes IRF-5 as a novel therapeutic target to prevent loss of memory $CD4^+$ T cells in PLWH.

Keywords : CD4+ T cells, IRF5, HIV, apoptosis.

SOMMAIRE RÉCAPITULATIF

Les infections au virus de l'immunodéficience humaine de type 1 (VIH-1) demeurent un enjeu de santé publique majeur. Comparativement à de nombreux virus, le corps humain ne peut se débarrasser complètement du VIH, même avec l'aide du ART, ce qui en fait une infection que les patients conservent toute leur vie durant (Chun *et al.*, 1997).

L'une des caractéristiques de l'infection par le VIH est la perte progressive des lymphocytes T $CD4^+$, venant affecter à la fois les cellules infectées et les cellules avoisinantes non-infectées. La mort des cellules $CD4^+$T se produit par le biais de plusieurs mécanismes, notamment la cytopathogénicité virale directe, leur destruction par les lymphocytes T cytotoxiques $CD8^+$ et la pyroptose des lymphocytes T $CD4^+$, ou encore l'apoptose des cellules avoisinantes non-infectées, cette dernière étant en grande partie médiée par Fas/FasL (Doitsh *et al.*, 2014; Katsikis *et al.*, 1995; Massanella *et al.*, 2013). Le maintien d'un nombre critique de lymphocytes T $CD4^+$ est essentiel pour prévenir les infections opportunistes secondaires. Le ART contrôle efficacement la réplication virale et la majorité des VIH-1^+ sont en mesure de rétablir leur nombre de lymphocytes T $CD4^+$ grâce au traitement. Néanmoins, il n'est toujours pas compris pourquoi l'homéostasie des lymphocytes T $CD4^+$ ne se trouve pas totalement normalisée chez les PLWH sous ART. Plus important encore, les lymphocytes mémoires T $CD4^+$ de ces PLWH sont plus souvent sujettes à l'apoptose médiée par Fas (Dyrhol-Riise *et al.*, 2001; van Grevenynghe *et al.*, 2008b). Les mécanismes conduisant à cette sensibilité accrue ne sont que partiellement connus et impliquent l'activité proapoptotique du facteur de transcription Foxo3a (van Grevenynghe *et al.*, 2008b). Bien que la réplication virale se trouve sous contrôle, des niveaux résiduels d'activation immunitaire et d'inflammation persistent chez les PLWH sous ART (Finzi *et al.*, 1999; Wada *et al.*, 2015).

En recherchant les molécules et les voies impliquées dans le maintien des lymphocytes T $CD4^+$ dans des environnements inflammatoires chroniques, nous avons récemment identifié le facteur de transcription interferon regulatory factor 5 (IRF-5) en tant qu'acteur nuisible à la survie des lymphocytes T $CD4^+$ dans les cas de leishmaniose viscérale chronique (Fabié *et al.*, 2018). IRF-5 est un membre de la famille des facteurs de régulation des interférons (IRF), un groupe de facteurs de transcription revêtant divers rôles, notamment l'activation transcriptionnelle des interférons, ainsi que la modulation de la croissance et de la différenciation cellulaire, de l'apoptose et de l'activité du système immunitaire (Honda *et al.*, 2006a; Tamura *et al.*, 2008). IRF-5 a fait l'objet de beaucoup d'attention ces dernières années, en raison de son rôle dans la

régulation des réponses inflammatoires dans les maladies immunologiques. IRF-5 s'exprime de façon constitutive chez les cellules présentatrices d'antigènes, où il participe à l'activation des cytokines inflammatoires ainsi qu'à la production d'interférons de type I (IFN-I) (Barnes *et al.*, 2001; Barnes *et al.*, 2004; Takaoka *et al.*, 2005), et a été identifié comme marqueur de la polarisation des macrophages M1 (Krausgruber *et al.*, 2011). En outre, IRF-5 agit dans la suppression des tumeurs (Hu *et al.*, 2005) et est essentiel dans l'induction de l'apoptose en réponse aux lésions provoquées à l'ADN des cellules tumorales (Bi *et al.*, 2014b). IRF-5 intervient également dans l'apoptose en modulant les gènes proapoptiques, notamment DR5, Fas, Caspase 8, DAP kinase 2, Bak et Bax (Hu *et al.*, 2009). Dans les cellules B, IRF-5 induit l'activation, la prolifération ainsi que la différenciation des plasmoblastes (De *et al.*, 2017), et est nécessaire à la production d'anticorps et à la commutation isotypique (Fang *et al.*, 2012). Cependant, bien que IRF-5 semble nécessaire à un assemblage optimal du complexe de signalisation initié par le récepteur des cellules T (TCR) (Yan *et al.*, 2020), son rôle dans les cellules T n'est pas encore entièrement compris.

Nous avons également démontré que IRF-5 s'exprime de manière croissante au cours d'une infection chronique à *Leishmania donovani* chez la souris et pouvait s'activer par l'intermédiaire du TLR7, exprimé dans les cellules T. Fait intéressant, par l'intermédiaire de TLR7, les cellules T IFNγ^{+} CD4^{+} étaient capables de détecter le matériel cellulaire apoptotique issu de dommage tissulaire. Ces résultats suggèrent que la détection des motifs moléculaires associés aux dommages (DAMP) libérés à la suite d'une lésion tissulaire dans un environnement inflammatoire chronique et l'augmentation concomitante du TLR7 par les cellules T CD4^{+} peuvent favoriser la mort cellulaire. L'infection par le VIH-1 se caractérisant par un environnement fortement inflammatoire, une perturbation des tissus, une régulation à la hausse du TLR7 dans les lymphocytes T CD4^{+} ainsi qu'un déclin progressif de leur fonction (McCune, 2001b), nous nous sommes interrogés si la voie TLR7-IRF5 était impliquée dans l'altération de l'homéostasie des lymphocytes mémoires T CD4^{+} lors d'une infection au VIH-1.

Ainsi, nous avons démontré que IRF-5 présentait des niveaux d'expression et d'activation plus élevés et que TLR7 était régulé à la hausse dans les lymphocytes T CD4^{+} des VIH-1^{+} par rapport aux CE et aux VIH-1-. En outre, le déclenchement du TLR7 a favorisé l'expression du IRF-5, qui à son tour a induit l'expression de la caspase 8 dans les lymphocytes T CD4^{+} des VIH-1^{+}, mais pas chez les personnes VIH-1-, sensibilisant ces cellules à l'apoptose médiée par Fas. Ce processus de mort cellulaire a pu être arrêté à l'aide de peptides de pénétration cellulaire bloquant IRF-5. Nous proposons donc que l'expression accrue du TLR7 et du IRF-5 dans les cellules

mémoire T CD4$^+$ des PLWH sous ART représente une empreinte venant affecter l'homéostasie des lymphocytes mémoires T CD4$^+$.

Nous avons précédemment montré que l'activation du facteur de transcription IRF-5 dans les cellules murines T CD4$^+$ IFN-γ$^+$ entraîne la mort cellulaire lors de la leishmaniose viscérale chronique (Fabié *et al.*, 2018). Fait intéressant, IRF-5 était induit suite à une stimulation du TLR7 par du matériel cellulaire apoptotique dérivé de la perturbation tissulaire inflammatoire, suggérant que la détection de DAMP par les lymphocytes T CD4$^+$ pourrait ne pas être spécifique qu'aux microbes. Par conséquent, nous nous demandions si l'IRF-5 est également exprimé et favorise la mort cellulaire dans les cellules humaines T CD4$^+$ dans le contexte d'infection par le VIH. Nous avons choisi d'analyser la fonction du IRF-5 dans les lymphocytes T CD4$^+$ des PLWH car l'infection est associée à un environnement inflammatoire intense et à une perte de lymphocytes T CD4$^+$ avant le début du ART (phase PHI), et parce que le TLR7 est exprimé dans les lymphocytes T CD4$^+$ lors d'infection par le VIH-1 (Dominguez-Villar *et al.*, 2015).

Nous avons observé une proportion plus élevée de lymphocytes T CD4$^+$ exprimant l'IRF-5 chez les participants PHI par rapport aux lymphocytes T CD4$^+$ des participants témoins VIH-1$^-$.De plus, ce facteur de transcription était co-localisé avec le noyau dans environ 40 à 60 % des lymphocytes T CD4$^+$ exprimant l'IRF-5 chez les PLWH par rapport à 1,8 à 6,7 % dans les lymphocytes T CD4+ des participants témoins VIH-1-. De manière similaire à ce que nous avons précédemment observé chez les souris (Fabié *et al.*, 2018), la majorité des cellules exprimant l'IRF-5 sécrétaient également de l'IFN-γ et étaient principalement des cellules mémoires et effectrices. Tel qu'attendu, il se trouvait également plus de cellules AnnexinV$^+$ parmi les lymphocytes T CD4$^+$ des PLWH, un marqueur courant de la mort cellulaire et de l'apoptose, par rapport aux cellules des participants témoins VIH-1-. Notamment, l'expression de l'IRF-5 était positivement corrélée à l'expression de l'AnnexinV et l'expression de l'AnnexinV était inversement corrélée au nombre de lymphocytes T CD4$^+$ chez les PLWH. De plus, l'expression de l'IRF-5 était également inversement corrélée au nombre de lymphocytes T CD4$^+$, suggérant que ce facteur de transcription pourrait contribuer à la mort cellulaire.

Les patients VIH-1$^+$ suivant un ART présentent toujours un faible niveau d'inflammation (Finzi *et al.*, 1999; Wada *et al.*, 2015), et leurs lymphocytes mémoires T CD4$^+$ sont susceptibles à l'apoptose médiée par Fas, malgré des années d'avirémie dans le compartiment sanguin (Dyrhol-Riise *et al.*, 2001; van Grevenynghe *et al.*, 2008b). Ainsi, nous voulions savoir si l'IRF-5 est également exprimé dans les lymphocytes mémoires T CD4$^+$ des PLWH suivant un ART et s'il est impliqué dans la sensibilisation à l'apoptose des lymphocytes mémoires T CD4$^+$.

PLWH et suivant un ART qui ont participé à cette étude ont présenté à la fois une suppression virale (charges virales < 40 copies/ml) et un rétablissement des lymphocytes T CD4$^+$ (> 400 cellules/µl dans le sang après le traitement) pendant au moins 5 ans. Sans recevoir de traitement, les EC présentaient des charges virales indétectables (< 40 copies/ml) pendant plus de 5 ans, avec des comptes de lymphocytes T CD4$^+$ supérieurs à 400 cellules/µl dans leur sang. Nous avons découvert une présence significativement plus élevées de lymphocytes T CD4$^+$ exprimant l'IRF-5 chez PLWH suivant un ART en comparaison au groupe témois. De plus, l'IRF-5 était co-localisé avec le noyau dans près de la moitié des cellules IRF-5$^+$. Étant donné que les CE VIH-1$^+$ peuvent contrôler leur charge virale plasmatique tout en maintenant leur nombre de lymphocytes T CD4$^+$ en l'absence de ART, nous étions intéressés à déterminer le pourcentage de lymphocytes T CD4$^+$ isolées exprimant l'IRF-5 chez les donneurs EC. Contrairement aux PLWH suivant un ART, les CE présentaient un nombre plus faible de cellules IRF-5$^+$ T CD4$^+$, l'IRF-5 étant actif dans environ 20-25 % des cellules IRF-5$^+$. Indépendamment des niveaux d'expression, l'IRF-5 était présent principalement dans les cellules mémoires et effectrices T CD4$^+$ dans les trois groupes d'étude. Comme l'IRF-5 est exprimée dans les lymphocytes mémoires T CD4$^+$ et que ces cellules sont plus susceptibles à l'apoptose chez les patients atteints du PLWH et suivant un ART, nous avons décidé de porter notre attention sur les lymphocytes mémoires T CD4$^+$.

Ensuite, le pourcentage de cellules apoptotiques dans les différents groupes d'étude a été comparé. L'apoptose initiale a été déterminée par la présence de cellules positives à l'annexine V et à la caspase-3 clivée. Contrairement aux EC, les lymphocytes mémoires T CD4$^+$ des PLWH suivant un ART exprimaient des niveaux significativement plus élevés d'AnnexinV par rapport au groupe témoin non infecté, suggérant que la mort des lymphocytes mémoires T CD4$^+$ était plus fréquente chez les patients atteints du PLWH et suivant un ART. L'expression d'AnnexinV dans les lymphocytes T CD4$^+$ mémoires se correlait à l'expression d'IRF-5, suggérant que ce facteur de transcription pourrait contribuer à la mort des cellules mémoires chez les donneurs atteints du VIH-1$^+$ suivant un ART. Aucune corrélation n'a été trouvée dans les cellules mémoires des EC entre l'expression d'IRF-5 et d'Annexin V.

Nous souhaitions ensuite examiner les partenaires de signalisation en amont de l'IRF-5 dans les lymphocytes mémoires T CD4$^+$. Nous avions précédemment démontré que le TLR7 s'exprimait de façon croissante dans les lymphocytes T CD4$^+$ tout au long de l'infection par *Leishmania donovani* chez les souris et que la stimulation du TLR7 entraînait l'induction du IRF-5 (Fabié *et al.*, 2018). On sait que le TLR7 est exprimé dans les cellules T CD4$^+$ humaines au moment d'une infection par le VIH-1(Dominguez-Villar *et al.*, 2015), mais on ignore encore si l'expression du

TLR7 est maintenue dans les lymphocytes T CD4$^+$ suite à un ART. Ainsi, nous avons analysé l'expression de l'ARNm du TLR7 dans les lymphocytes T CD4$^+$ isolées des PLWH et suivant un ART, des CE et des individus VIH-1$^-$. Les niveaux les plus élevés d'expression de l'ARNm du TLR7 ont été détectés dans les lymphocytes T CD4+ des PLWH et suivant un ART, tandis que les CE exprimaient des niveaux intermédiaires de TLR7 par rapport aux donneurs VIH-1-. Ensuite, nous avons stimulé les lymphocytes T CD4$^+$ des différents groupes d'étude avec de l'imiquimod (IMQ), un agoniste du TLR7. Nous avons constaté que le traitement des cellules à l'aide de l'IMQ induisait l'expression de l'IRF-5 dans les lymphocytes mémoires T CD4$^+$ des PLWH suivant un ART et, dans une moindre mesure, des donneurs CE, mais pas chez les individus VIH-1$^-$.

En accord avec nos résultats obtenus sur les souris (Fabié *et al.*, 2018), le traitement par l'IMQ induit l'apoptose (« Zombie Aqua$^-$ AnnexinV$^+$ Caspase 3$^+$ ») et la mort cellulaire (« Zombie Aqua$^+$ AnnexinV$^+$ ») dans les lymphocytes mémoires T CD4$^+$ des PLWH sous ART à un degré significativement plus élevé que dans les lymphocytes mémoires T CD4$^+$ des CE, corrélant directement avec les niveaux d'expression du TLR7. À noter que le traitement par l'IMQ n'affecte pas la survie cellulaire des T CD4$^+$ chez les individus VIH-1$^-$. Nos résultats suggèrent que l'axe TLR7-IRF-5 pourrait jouer un rôle dans la mort des lymphocytes mémoires T CD4$^+$ chez les PLWH sous traitement ART. Cette hypothèse est étayée par la corrélation directe entre l'expression de TLR7 et IRF-5, ainsi qu'entre l'AnnexinV et l'expression du TLR7. Bien que TLR7 et IRF5 soient également exprimés dans les T CD4$^+$ des EC, aucune corrélation n'a été trouvée entre l'expression d'IRF-5 et TLR7, ou TLR7 et AnnexinV dans ces cellules.

De nombreuses molécules impliquées dans la mort cellulaire ont été décrites comme étant des cibles moléculaires en aval d'IRF-5, notamment Fas, DR5 et caspase 8 (Hu *et al.*, 2009). Comme il a été rapporté précédemment que la mort des lymphocytes mémoires T CD4$^+$ lors de l'infection par le VIH-1 est médiée par Fas (van Grevenynghe *et al.*, 2008b), et que l'IRF-5 est nécessaire à l'apoptose induite par Fas (Couzinet *et al.*, 2008), nous avons entrepris d'étudier si Fas se trouvait en aval d'IRF-5 dans les lymphocytes T CD4$^+$ humains. En accord avec la littérature (van Grevenynghe *et al.*, 2008b), nous n'avons trouvé aucune différence dans l'expression de Fas entre les groupes étudiés. Par conséquent, nous avons examiné si l'expression de lacCaspase 8 différait entre les différents groupes d'étude. Une surexpression significative des niveaux d'ARNm de CASP8 a été observée dans les lymphocytes T CD4$^+$ de PLWH sous ART par rapport aux EC et individus VIH-1$^-$. De plus, lorsque nous avons traité les lymphocytes T CD4$^+$ avec l'IMQ, les lymphocytes T CD4$^+$ mémoires de PLWH sous ART, mais pas celles des participants VIH-1$^-$, ont

régulé à la hausse l'expression de l'ARNm de CASP8. Dans l'ensemble, nos résultats suggèrent que l'axe TLR7-IRF-5 induit l'expression de la caspase 8 et semble être actif dans les lymphocytes T $CD4^+$ mémoires de PLWH sous ART.

Il a été démontré que l'IRF-5 est nécessaire à la mort cellulaire médiée par Fas. Nous avons donc étudié si l'interaction Fas/FasL pouvait également induire l'IRF-5. Lorsque nous avons traité les lymphocytes T $CD4^+$ avec le ligand Fas recombinant (rFasL : « recombinant human Fas ligand ») humain, nous avons observé des pourcentages plus élevés de lymphocytes mémoires T $CD4^+$IRF-5^+ chez les individus VIH-1^- et PLWH sous ART. Comme l'IRF-5 et la caspase 8 font également partie de la voie de signalisation Fas/FasL (Couzinet *et al.*, 2008; Hitoshi *et al.*, 1998; Zhang *et al.*, 1998), nous avons émis l'hypothèse que l'activation de l'axe TLR7-IRF-5-caspase 8 prédisposerait les cellules à l'apoptose médiée par Fas et renforcerait la mort cellulaire. Pour prouver notre hypothèse, nous avons prétraité les cellules avec ou sans IMQ pendant 18 heures, suivi d'une incubation de 12 heures avec rFasL, puis analysé la fréquence des lymphocytes T $CD4^+$ mémoires apoptotiques et morts. Comme prévu, nous avons détecté des fréquences significativement plus élevées de cellules apoptotiques et de cellules mortes dans les lymphocytes T $CD4^+$ chez les PLWH sous ART par rapport aux VIH-1^-, suivant une incubation avec le rFasL seulement, suggérant que les lymphocytes T $CD4^+$ chez les PLWH sous ART présentent une prédisposition intrinsèquement plus élevée à mourir par apoptose médiée par Fas que les lymphocytes T $CD4^+$ VIH-1^-. Nous avons également observé un effet de synergie dans l'induction des cellules apoptotiques et de la mort cellulaire, après avoir traité les lymphocytes T $CD4^+$ purifiées avec de l'IMQ, et ensuite du rFasL, comparativement aux cellules incubées avec du rFasL seulement. Cet effet était moins prononcé chez les T $CD4^+$ VIH-1^- que chez les PLWH sous ART. Nous avons ensuite analysé les niveaux d'ARNm de la CASP8 par RTqPCR et constaté une synergie dans l'induction de l'ARNm de la CASP8 dans les lymphocytes T $CD4^+$ de PLWH sous ART, mais uniquement lors de la stimulation avec IMQ suivie du rFasL et non avec le rFasL seul. Dans l'ensemble, nos résultats montrent que la voie TLR7-IRF-5 se connecte à la voie de signalisation Fas/FasL, amplifiant ensuite l'expression d'IRF-5 et de la caspase 8, créant ainsi un cycle menant à la mort cellulaire.

Les résultats obtenus jusqu'à présent indiquent que l'IRF-5 est une molécule clé à la fois dans l'axe TLR7-IRF-5 et dans la voie de signalisation Fas/FasL. Par conséquent, nous avons cherché à bloquer l'activation de l'IRF-5 pour limiter la mort cellulaire. À cette fin, nous avons décidé d'utiliser des peptides inhibiteurs de l'IRF-5 développés par Banga *et al.* (Banga *et al.*, 2020). Ces peptides cellulaires perméables (IRF-5-CPP) bloquent efficacement la fonction de l'IRF-5 en aval

de la phosphorylation d'IRF-5, empêchant son activation subséquente de l'IRF-5 dans divers types cellulaires; cependant, les IRF-5-CPP n'ont pas encore été testés dans les cellules T. Ainsi, nous avons choisi quatre peptides différents (P1-4) avec des spécificités cellulaires différentes. Nous avons d'abord testé la cytotoxicité de différents IRF-5-CPP, y compris le peptide témoin, sur les cellules humaines T $CD4^+$ de donneurs VIH-1^-, à des concentrations variant de 0 à 50 µM. Les IRF-5-CPP se sont révélés non cytotoxiques jusqu'à une concentration de 20 µM. Lorsque nous avons testé la capacité des IRF-5-CPP à inhiber la localisation nucléaire de l'IRF-5 humain dans les lymphocytes T $CD4^+$ non traitées et stimulées par l'IMQ de donneurs VIH-1^- et PLWH sous ART, nous avons observé que P1 et P3 étaient les peptides les plus efficaces pour prévenir l'activation de l'IRF-5 dans les lymphocytes T $CD4^+$ non stimulées ainsi que dans les cellules traitées à l'IMQ. Nous avons également testé si P1 et P3 pouvaient être utilisés ensemble pour obtenir un blocage plus fort de l'IRF-5 et finalement inhiber la mort cellulaire induite par l'IMQ. À cette fin, les lymphocytes T $CD4^+$ des donneurs VIH-1^- et PLWH sous ART ont été traitées soit avec 5 µM de P1 ou P3 seul, soit avec un mélange de P1 et P3, puis stimulées par l'IMQ. le mélange P1/P3 était légèrement plus efficace pour bloquer l'activation de l'IRF-5 dans les lymphocytes T $CD4^+$ atteintes du PLWH sous ART lors de la stimulation par l'IMQ. En effet, nous avons observé une inhibition de 44% de la localisation nucléaire de l'IRF-5 après traitement avec P1, 40% après incubation avec P3 et environ 60% lorsque les cellules étaient co-cultivées avec P1 et P3. Ainsi, nous avons décidé d'utiliser une combinaison de P1 et P3 pour inhiber la voie TLR7-IRF-5; le peptide témoin a été utilisé pour tous les groupes contrôles « Peptideneg ». À cette fin, nous avons traité les lymphocytes T $CD4^+$ purifiés avec le mélange de peptides 30 minutes avant la stimulation par l'IMQ. La combinaison des deux IRF-5-CPPs a bloqué l'apoptose de façon significative dans les lymphocytes mémoires T $CD4^+$ des PLWH sous ART. En effet, nous avons observé des fréquences plus faibles de cellules apoptotiques et de cellules mortes dans les lymphocytes T $CD4^+$ mémoires de PLWH sous ART après stimulation par l'IMQ. Comme prévu, ce n'était pas le cas chez les donneurs VIH-1^-, car ces cellules expriment des niveaux très faibles de TLR7 et ne répondent pas à l'IMQ. Des résultats similaires ont été obtenus lorsque nous avons analysé les niveaux d'ARNm de CASP8, qui étaient significativement réduits lorsque nous avons ajouté des peptides bloquants l'IRF-5 aux lymphocytes T $CD4^+$ purifiées de patients PLWH stimulées par l'IMQ .

Comme la voie Fas/FasL est impliquée dans la mort des lymphocytes mémoires T $CD4^+$ lors de l'infection par le VIH-1, nous avons ensuite évalué si le mélange d'IRF-5-CPP pouvait bloquer

l'apoptose médiée par Fas dans les lymphocytes mémoires T CD4$^+$ de PLWH sous ART. Les lymphocytes T CD4$^+$ ont été incubés pendant 1 heure avec 5 µM d'un mélange de P1 et P3 ou du peptide témoin, suivi d'une incubation de 18 heures avec ou sans IMQ. Les cellules ont ensuite été traitées avec du rFasL pendant 12 heures supplémentaires. Remarquablement, le mélange d'IRF-5-CPP pouvait limiter significativement l'apoptose médiée par Fas. En fait, l'augmentation de la fréquence de cellules apoptotiques ou de cellules mortes après traitement par rFasL ou IMQ + rFasL a été réduite d'environ la moitié suite au blocage de l'IRF-5 dans les lymphocytes T CD4$^+$ de PLWH. Un effet similaire, mais moins prononcé, a été observé dans les lymphocytes T CD4$^+$ de donneurs VIH-1$^-$. Aucune différence n'a été observée entre les lymphocytes T CD4$^+$ de donneurs masculins et féminins, indépendamment de leur statut, qu'ils soient PLWH ou VIH-1.

Enfin, pour prouver clairement que l'inhibition de l'apoptose médiée par Fas dépendait du blocage de l'IRF-5, nous avons évalué les niveaux d'ARNm de CASP8 après les différents traitements, la caspase 8 étant une cible moléculaire en aval de l'IRF-5. Nous avons observé une réduction d'environ 50% de l'induction de l'ARNm de CASP8 dans les lymphocytes T CD4$^+$ de patients VIH-1+, traitées soit avec du rFasL, soit avec de l'IMQ + rFasL après le blocage de l'IRF-5. L'effet dans les lymphocytes mémoires T CD4$^+$ de donneurs VIH-1$^-$ était seulement partiel et moins prononcé que celui observé dans les lymphocytes T CD4$^+$ de PLWH. Aucune différence n'a été observée entre les lymphocytes T CD4$^+$ de donneurs masculins et féminins. Dans l'ensemble, nos résultats suggèrent que l'IRF-5 joue un rôle central dans la mort des lymphocytes mémoires T CD4$^+$ chez les patients PLWH sous ART, non seulement en tant que cible en aval de TLR7, mais aussi en étant directement impliqué dans l'apoptose médiée par Fas.

Bien que l'expression de TLR7 sur les cellules T ait été rapportée chez les souris et les humains, les voies de signalisation favorisant la surexpression de TLR7 dans les lymphocytes T CD4$^+$ demeurent inconnues. Il a été démontré que le IFN de type I renforce l'expression des TLR dans les cellules B (Doucett *et al.*, 2005; Silva-Barrios *et al.*, 2016b; Thibault *et al.*, 2009). Comme l'IFN-ß fait souvent partie des environnements inflammatoires chroniques associés aux infections persistantes (Dillon *et al.*, 2018) et que TLR7 est surexprimé dans les lymphocytes T CD4$^+$ au cours des infections persistantes (Fabié *et al.*, 2018), nous avons d'abord évalué si les lymphocytes T CD4$^+$ purifiées de PLWH exposées à de l'IFN-ß recombinant surexprimaient TLR7. Nous avons constaté que l'IFN-ß favorisait la surexpression de l'ARNm de TLR7 6 heures après l'exposition initiale et que cette expression était maintenue sur 24 heures. La perturbation tissulaire et la libération de DAMP sont également caractéristiques des environnements inflammatoires chroniques. Ainsi, nous avons exposé les lymphocytes T CD4$^+$ de donneurs VIH-

1- à du matériel cellulaire apoptotique. De manière intéressante, le matériel cellulaire apoptotique pouvait également augmenter l'expression de l'ARNm de TLR7, bien que dans une moindre mesure que l'IFN-ß ; néanmoins, son effet était synergique avec l'IFN-ß. Cela suggère que les lymphocytes T $CD4^+$ dans un environnement inflammatoire peuvent surexprimer TLR7 indépendamment de leur spécificité antigénique. Ensuite, nous avons évalué si le primage des lymphocytes T $CD4^+$ en présence de DAMP et d'IFN-ß pouvait moduler les niveaux d'expression de l'ARNm de TLR7. La stimulation avec un anti-CD3/CD28 en présence ou en l'absence de matériel cellulaire apoptotique induisait légèrement l'expression de TLR7. Cependant, la stimulation avec un anti-CD3/CD28 des cellules exposées précédemment à l'IFN-ß favorisait fortement l'expression de TLR7, ce qui était encore renforcé en présence de DAMPs, indiquant que les lymphocytes T $CD4^+$ spécifiques de l'antigène amorcées dans un environnement inflammatoire persistant riche en IFN-ß et en DAMPs sont destinées à exprimer des niveaux élevés de TLR7.

Pour vérifier si les lymphocytes T $CD4^+$ exposées à l'IFN-ß et/ou au matériel cellulaire apoptotique, avec ou sans stimulation anti-CD3/CD28, étaient plus susceptibles à l'apoptose médiée par Fas, nous avons traité les cellules avec du rFasL. Lorsque nous avons analysé les lymphocytes T $CD4^+$ mémoires, nous avons trouvé des fréquences plus élevées de cellules IRF-5^+, de cellules apoptotiques, et de cellules mortes lorsque les lymphocytes T $CD4^+$ étaient exposées à l'IFNß + DAMPs avant le traitement par rFasL, par rapport à rFasL seul. La stimulation avec un anti-CD3/CD28 n'a pas modifié la susceptibilité des cellules à l'apoptose médiée par Fas, mais a légèrement augmenté l'expression d'IRF-5. En accord avec les niveaux d'expression de TLR7, les lymphocytes T $CD4^+$ exposés à l'IFNß + DAMPs avant la stimulation avec un anti-CD3/CD28 étaient significativement plus susceptibles à l'apoptose médiée par Fas et exprimaient les niveaux les plus élevés d'IRF-parmi les groupes de traitement. Des résultats similaires ont été obtenus lorsque nous avons analysé les lymphocytes T $CD4^+$ mémoires. Dans l'ensemble, ces résultats suggèrent que l'exposition de lymphocytes T $CD4^+$ non spécifiques ou de lymphocytes T $CD4^+$ spécifiques de l'antigène à du matériel apoptotique et à l'IFN-ß favorise la surexpression de TLR7 et prédispose les cellules à une apoptose médiée par FAS.

En résumé, nous proposons que les lymphocytes mémoires T $CD4^+$ de patients PLWH sous ART expriment TLR7 et IRF-5, ce qui représente une empreinte prédisposant ces cellules à une apoptose médiée par Fas. L'axe TLR7-IRF-5 peut être activé par les DAMPs, entraînant une surexpression de caspase 8. Cette voie alimente directement la voie Fas-FasL, augmentant l'expression d'IRF-5 et de caspase 8 conduisant à la mort cellulaire. En revanche, les lymphocytes

T $CD4^+$ mémoires de donneurs $VIH\text{-}1^-$ et des CE expriment de faibles niveaux de TLR7 et d'IRF-5, ce qui rend ces cellules moins à même de détecter les DAMP et finalement moins enclines à l'apoptose médiée par Fas.

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A

Accidental cell death (ACD)

Activation domain (AD)

Autophagy-dependent cell death (ADCD)

Acquired Immune Deficiency Syndrome (AIDS)

Absent in melanoma 2 (AIM2)-like receptors (ALRs)

Apoptotic material (AM)

Apoptotic protease-activating factor 1 (APAF1)

Autoinhibitory region (AR)

Antiretroviral therapy (ART)

Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC)

Adult T cell leukemia (ATL)

Adenosine triphosphate (ATP)

Azidothymidine (AZT)

B

BCL-2 antagonist/killer (BAK)

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β-D-glucan (BDG)

BH3-interacting domain death agonist (BID)

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D

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Death-associated protein kinase 1 (DAPK1)

DNA binding domain (DBD)

Dendritic cell (DC)

DCC netrin 1 receptor

Dendritic cell immunoreceptor (DCIR)

Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN)

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Death-inducing signaling complex (DISC)

Death receptor 5 (DR5)

Double-stranded RNA (dsRNA)

E

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Elite controllers (EC)

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Endoplasmic reticulum (ER)

F

Fas-related death domain protein (FADD)

Forkhead box transcription factor O class 3a (Foxo3a)

Formyl Peptide Receptor 1 (FPR1)

Fonds de la Recherche du Quebec-Santé (FRQ-S)

G

Group-specific antigen (Gag)

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Melanoma Differentiation-Associated Protein 5 (MDA5)

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Muramyl dipeptide (MDP)

Macrophage inflammatory protein 1α (MIP-1α) and 1β (MIP-1β)

Mixed-lineage kinase domain-like protein (MLKL)

mitochondrial membrane potential (MMP or ΔΨm)

Matrix Metalloproteinase-3 (MMP-3)

Mitochondrial outer membrane permeabilization (MOMP)

Mitochondria DNA (mtDNA)

Mitochondria DAMPs (MTDs)

Murine IRF-5 (MuIRF-5)

Myeloid differentiation primary response 88 (MyD88)

N

neutralizing antibodies (NAbs)

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Negative factor (nef)

Nuclear export signal (NES)

nuclear factor kappa B (NF-κB)

Natural killer (NK)

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Viral infectivity factor (vif)

Visceral leishmaniasis (VL)

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Viral protein U (vpu)

W

World Health organization (WHO)

Z

Zymosan (ZMS)

1 CHAPTER 1 INTRODUCTION

PART I: CD4$^+$ T CELLS DURING CHRONIC INFECTION

CD4$^+$ T cells play a multifaceted role in the immune response during chronic infections. Their functions are critical for maintaining a balance between controlling the infection and preventing excessive immune-mediated tissue damage (Kervevan *et al.*, 2021). One of the key roles of CD4$^+$ T cells during chronic infections is to keep a sustained immune activation. CD4$^+$ T cells help sustain the immune response by providing long-lasting help to other immune cells, including CD8$^+$ T cells, B cells, and macrophages (Kim *et al.*, 2019; Raziorrouh *et al.*, 2016; Snell, 2022; Zander *et al.*, 2019). This support is crucial for maintaining immune surveillance over extended periods.

CD4$^+$ T are also involved in the maintenance of immunological memory during chronic infections (Snell, 2022). Indeed, CD4$^+$T cells support the maintenance of memory T cell populations and promote the formation of memory B cells (Hale *et al.*, 2013; Krueger *et al.*, 2021). Moreover, CD4$^+$ T cells provide essential help to B cells, facilitating the production of antibodies that can aid in neutralizing pathogens and controlling the infection (Raziorrouh *et al.*, 2016; Schweier *et al.*, 2019). Neutralizing antibodies offer defense against numerous viral infections, and CD4$^+$ T cells play a role in enhancing B cells' ability to produce more potent and durable antibody reactions (Sarkar *et al.*, 2021; Wang *et al.*, 2022).

Another important role of CD4$^+$ T cells during chronic infections is the regulation of tolerance. Tregs are a subset of CD4$^+$ T cells that play a role in regulating immune responses and preventing excessive inflammation (Ceulemans *et al.*, 2016; Hu *et al.*, 2017; Padovan, 2017; Presicce *et al.*, 2011; Thornton *et al.*, 1998). They help maintain immune tolerance and may modulate responses during chronic infections to prevent immune-mediated tissue damage (Laidlaw *et al.*, 2015; Shen *et al.*, 2016; Utzschneider *et al.*, 2016). Additionally, CD4$^+$ T cells can promote the resolution of inflammation after the acute phase of infection, preventing tissue damage caused by prolonged immune activation (Andreatta *et al.*, 2022; Xia *et al.*, 2022).

Overall, CD4$^+$ T cells play a crucial role in orchestrating and regulating the immune response during chronic infections, helping to control the infection while minimizing collateral damage to the host tissues (Feng *et al.*, 2007; Hu *et al.*, 2017; Padovan, 2017).

However, chronic infections, such as HIV and visceral leishmaniasis, involve a continuous immune response and can lead to gradual tissue damage, inflammation and disease progression over time. During chronic infection, high levels of inflammation persist, influencing the functions of T cells and amplifying both their exhaustion and cell death (Dondelinger *et al.*, 2019; Kuriakose *et al.*, 2016; Martin *et al.*, 2021; Salimzadeh *et al.*, 2018; Yong *et al.*, 2018). During chronic or prolonged infections, $CD4^+$ T cells can become exhausted due to prolonged activation and exposure to inflammatory signals. Exhausted $CD4^+$ T cells lose their functional capacity and can undergo cell death due to impaired signaling pathways or increased vulnerability to apoptosis (Crawford *et al.*, 2014; Jones *et al.*, 2008; McKinney *et al.*, 2015). This decline in T cell immunity in an inflammatory environment is a cause of morbidity and mortality in infectious diseases (Gao *et al.*, 2022). That is why the maintenance of functional memory $CD4^+$ T is of vital importance during chronic infections.

PART II: MECHANISMS OF CELL DEATH

Cell death refers to the process in which individual cells cease to function and eventually disintegrate or are removed from the body. This process is a natural and essential part of the life cycle of cells and plays several crucial roles in essential biological processes like the elimination of unneeded structures or cells, the control of cell populations, restructuring of tissues, formation of organs and maintaining the overall health and homeostasis of an organism (Nirmala *et al.*, 2020).

Cell death can be fundamentally divided into accidental cell death (ACD) and regulated cell death (RCD) based on their functional characteristics. ACD occurs as a chaotic response to extreme stress, while regulated cell death RCD is a controlled process characterized by the involvement of specific molecular and genetic pathways that serves vital roles in physiological functions (Galluzzi *et al.*, 2018).

1.1 Regulated cell death (RCD)

RCD is controlled by a complex network of interlinked signaling pathways and molecular mechanisms that can be initiated by internal disruptions or by the external cellular environment when the mechanisms that typically respond to stress are ineffective (Santagostino *et al.*, 2021).

Cells undergoing RCD are participating in a sequence that remains reversible until they reach a critical, irreversible phase. Various factors have been suggested to represent this threshold, including extensive caspase activation, loss of mitochondrial membrane potential (MMP or ΔΨm), full permeabilization of the mitochondrial outer membrane, or the exposure of phosphatidylserine residues that send out signals to neighboring normal cells, signifying a need for phagocytoses or clearance (Kroemer *et al.*, 2009).

RCD can occur in two distinct scenarios. First, during processes where cells naturally die as a part of the organism's normal life cycle, it is referred to as programmed cell death (PCD). In this case, no external factors are required to trigger cell death. However, when cells are unable to rectify errors resulting from disruptions in their normal intracellular programs, such as the cell cycle or proliferation, or when exposed to external toxic influences, the genetically predetermined RCD program is activated. This type of RCD includes various forms of canonical RCD, such as apoptosis, and non-apoptotic or non-canonical RCD, which includes necrotic cell death, autophagy-dependent cell death (ADCD), Lysosome-dependent cell death, pyroptosis, pyronecrosis, necroptosis, ferroptosis, PAN-optosis, and immunogenic cell death (ICD) (Galluzzi *et al.*, 2018)

1.1.1 Programmed cell death (PCD)

Currently, PCD is classified as a specific type of RCD that exclusively takes place in normal physiological situations, meaning it is not connected to disruptions in homeostasis and, therefore, does not occur in response to the inability to adapt to stress. PCD is considered the natural form of RCD responsible for embryonic, post-embryonic development and the maintenance of adult tissue. It is important to clarify that the terms 'PCD' and 'apoptosis' should not be used interchangeably, as cell death in the context of physiological development can exhibit characteristics that are distinct from apoptosis (Kopeina *et al.*, 2022).

1.1.2 Apoptosis

The term apoptosis was introduced in 1972 (Kerr *et al.*, 1972). Apoptosis relies on the activation of the cysteine-aspartic protease or cysteine-dependent aspartate-directed protease (caspase) family, without causing mitochondrial, lysosomal, or cell membrane damage, and without the release of cellular contents, thereby avoiding any inflammatory response (Elmore, 2007).

Caspases involved in apoptosis cleave cellular substrates, disassemble cellular components, break down structural proteins, resulting in cytoplasm condensation, chromatin compaction, DNA

fragmentation, all while maintaining the overall integrity of the plasma membrane in terms of morphology (Kesavardhana *et al.*, 2020).

Apoptosis is characterized by distinct morphological changes, including cell shrinkage, chromatin condensation, DNA fragmentation, and mitochondrial swelling. The cell nucleus undergoes fragmentation, and the cell itself divides into multiple apoptotic bodies, each enclosed by its own cell membrane (Taylor *et al.*, 2008).

Apoptosis can be categorized into intrinsic or extrinsic based on the triggers or pathways that initiate the process (Figure 1.1). Intrinsic apoptosis, also known as mitochondrial-mediated apoptosis, is initiated from within the cell by intracellular signals, such as DNA damage, cellular stress, or loss of survival signals. These signals can lead to changes in the permeability of the mitochondrial membrane, release of pro-apoptotic factors, and activation of caspases, ultimately resulting in cell death. The intrinsic apoptosis pathway is governed by critical regulators, the anti-apoptotic BCL-2 proteins (Lossi, 2022). The relative proportions of BCL-2 family members, particularly the BCL-2/ BCL-2 associated X protein (BAX) ratio, are key determinants of pro-apoptotic and anti-apoptotic pathways. BAX and BCL-2 regulate apoptosis by forming homodimers or heterodimers: BAX triggers apoptosis when forming homodimers, while the formation of heterodimers between BAX and BCL-2 inhibits apoptosis (Ding *et al.*, 2014).

BH3-only proteins are a subgroup of the Bcl-2 family that contain a single Bcl-2 Homology 3 (BH3) domain, a short sequence critical for their pro-apoptotic activity. BH3-only proteins activate BAX or BCL-2 antagonist/killer (BAK) on the mitochondrial surface, inducing an allosteric change that allows them to oligomerize and create macropores in the membrane. This process contributes to mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome C into the cytoplasm. Cytochrome C binds to apoptotic protease-activating factor 1 (APAF1) to form an apoptosome, activating caspase 9, which then activates caspase 3, leading to apoptosis. Additionally, caspase 8 cleaves downstream targets, either directly activating caspases 3 and 7, or cleaving BH3-interacting domain death agonist (BID), a BCL-2 family molecule (Adrain *et al.*, 2006; Tait *et al.*, 2010).

Apart from cytochrome c, various other factors involved in initiating apoptosis are released from the mitochondria into the cytosol, such as SMAC, which exerts its function by inhibiting inhibitor of apoptosis proteins (IAPs), such as XIAP (Srinivasula *et al.*, 2001).

The extrinsic apoptosis pathway is also known as the death receptor mediated apoptosis pathway. In general, death receptors are members of the tumour necrosis factor (TNF) superfamily (TNFRSF) and include several members that use different adaptor proteins, which

facilitates the interaction with the downstream multiprotein complexes. When death receptors are activated by ligand binding, they facilitate the formation of intricate multiprotein complexes within the intracellular domain of the receptor. These complexes are known as the "death-inducing signaling complex" (DISC) and control the activation and functions of caspases (Ashkenazi, 2002).

This extrinsic pathway can be categorized into two distinct types based on the downstream cascade reactions it triggers (Walczak *et al.*, 2008). The first type of apoptosis cascade is mediated by TNFRSFs members that use Fas-related death domain protein (FADD), including Fas, (TNFRSF10A (also referred to as TRAILR1or DR4), and TNFRSF10B (also referred to as TRAILR2 or DR5). For example, the binding of Fas ligand to Fas or TRAIL to DR4 or DR5 recruits FADD in the cytoplasm, leading to the activation of caspase 8 and the initiation of apoptosis (Chinnaiyan *et al.*, 1995).

The second type involves the apoptosis cascade initiated by TNFRSF member 1A (TNFRSF1A), which recruits as a platform adaptor TNFR-associated death domain (TRADD). TNF binding to TNFR1 leads to the recruitment of early complexes composed of TRADD and receptor-interacting serine/threonine protein kinase 1 (RIPK1). This triggers a series of subsequent signaling cascades that ultimately activate the initial caspases (caspase 8 or 10) to cause apoptosis (Vanamee É *et al.*, 2018).

Apoptosis can be induced by receptors other than TNFRSF. Dependence receptors represent a diverse family of membrane receptors united not by structural similarities, but by shared functional characteristics. Their most prominent feature is their dual capacity to initiate two contrasting signaling pathways. Contrary to TNFRSF, when dependence receptors bound to their ligands, these receptors activate conventional signaling pathways associated with cell survival, migration, and differentiation. However, when deprived of ligands, they do not remain quiescent; instead, they evoke an apoptotic signal. For example, dependence receptors, such as the unc-5 netrin receptor B (UNC5B) and the DCC netrin 1 receptor, have the potential to trigger the extrinsic apoptosis pathway (Ahn *et al.*, 2020). This occurs either through the activation of the initiator caspase 9 or the dephosphorylation of death-associated protein kinase 1 (DAPK1, also known as DAPK), especially upon the removal of their respective ligands. Consequently, cells expressing these receptors rely on the presence of ligands in the extracellular environment to ensure their survival (Bredesen *et al.*, 2004).

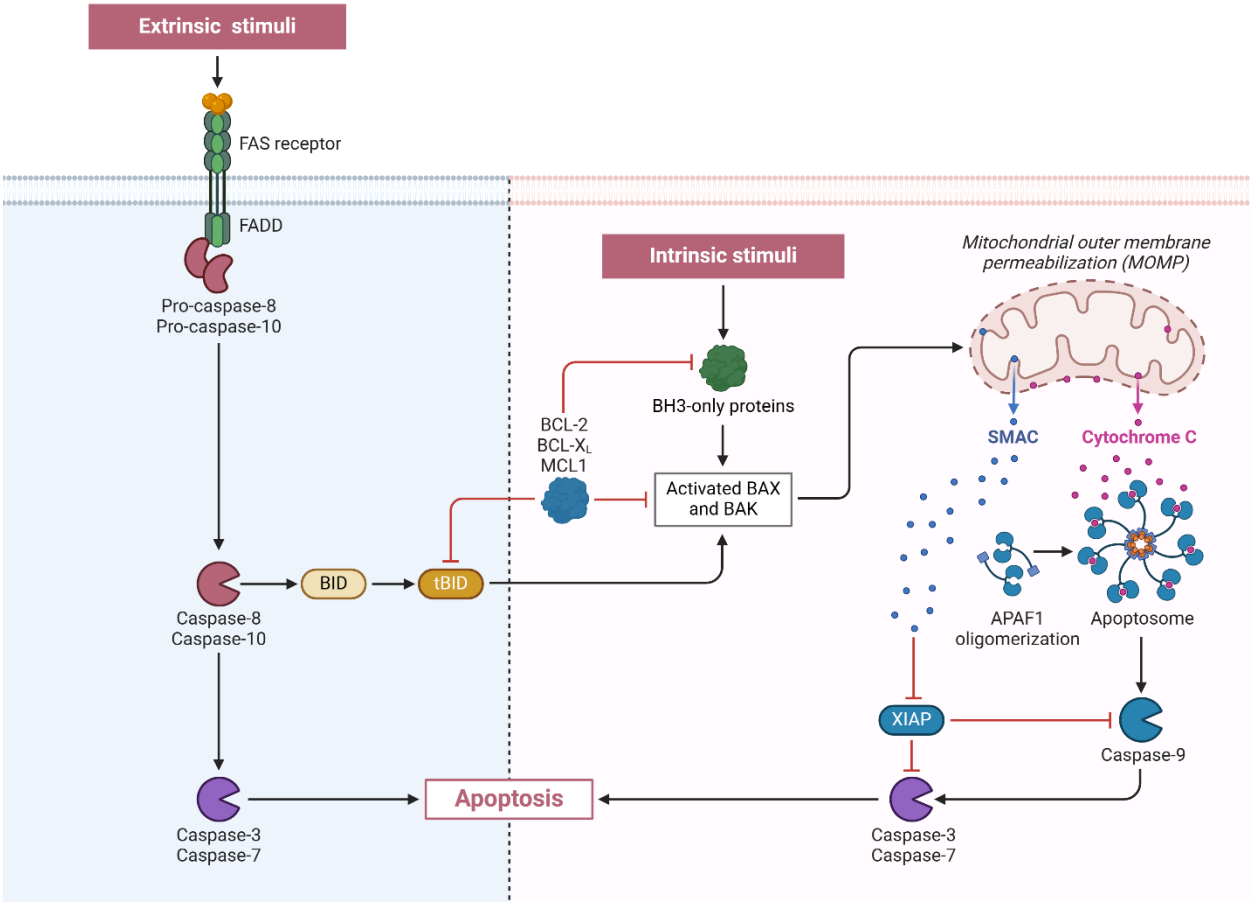

Figure 1.1 Apoptosis pathways

Apoptosis can be initiated through two distinct pathways: the extrinsic and intrinsic pathways. In the extrinsic pathway, external signals bind to death receptors on the cell surface (e.g., FAS receptor), triggering a caspase cascade that ultimately leads to cell death. Conversely, the intrinsic pathway is activated by internal stress signals, prompting mitochondria to release pro-apoptotic proteins such as SMAC and cytochrome C, which in turn activate caspases and induce apoptosis. Created with BioRender.com.

Additionally, apoptosis subtypes exist. For example, anoikis is triggered by the loss of cell-matrix or cell-cell interactions, when cells detach from their normal tissue environment (Frisch *et al.*, 1994). Endoplasmic reticulum (ER) stress can also trigger apoptosis in response to disturbances in protein folding and processing within the ER. This type of apoptosis is mediated by specific pathways, such as the unfolded protein response (UPR) and the ER-associated degradation pathway and can involve caspase activation and subsequent cell death (Tabas *et al.*, 2011).

While caspases are the main proteases involved in apoptosis, there are also caspase-independent pathways that can result in cell death. These pathways may involve other proteases, such as calpains or cathepsins, or other mechanisms, such as autophagy or necrosis, and can be triggered by various cellular stresses and damage (Nicotera, 2002).

1.1.3 Necrotic cell death or necrosis

Necrosis is marked by distinct morphological changes in cells, including an increase in cell volume known as oncosis, organelle swelling, rupture of the plasma membrane, and the subsequent release of intracellular contents into adjacent regions, triggering a chain reaction of inflammation and tissue injury (Majno *et al.*, 1995).

In contrast to apoptosis, necrosis is a type of cell death that does not rely on energy and occurs when a cell is severely and suddenly damaged by external factors like radiation, heat, chemicals, hypoxia, or other stressors, rendering it non-functional. Necrosis is rapid, catastrophic, and remains impervious to prevention through either pharmaceutical or genetic interventions (Edinger *et al.*, 2004).

Traditionally, necrosis is identified as an accidental, uncontrolled form of cell death. However, a growing body of evidence suggests that necrotic cell death can also be intricately regulated by a network of signal transduction pathways (Kroemer *et al.*, 2009).

1.1.4 Necroptosis

Necroptosis is the genetically regulated variant of necrosis (Degterev *et al.*, 2005). This controlled necrotic death mechanism is triggered by the activation of death receptors, such as TNFR1 and Fas, which induce the activation of RIPK1 (Holler *et al.*, 2000) RIPK1 and RIPK3 activate the mixed-lineage kinase domain-like protein (MLKL), leading to its oligomerization. When tumor necrosis factor (TNF) binds to tumor necrosis factor receptor 1 (TNFR1), it forms complex I (TNFR1, TRADD, RIPK1, TNFR associated factor 2 (TRAF2), cellular inhibitor of apoptosis proteins, and linear ubiquitin chain assembly complex), which regulates NF-κB activation and cell survival through ubiquitination processes. Stabilized RIPK1 in complex I recruits more factors, forming complex II (RIPK1/3, MLKL, caspase 8, FADD). If RIPK3 and MLKL are sufficient and caspase 8 is inhibited, complex II becomes the necrosome, increasing mitochondrial ROS, causing membrane permeabilization, and releasing intracellular contents (Grootjans *et al.*, 2017). Notably, caspase 8 acts as a negative regulator of this form of cell death. Moreover, the small

molecule inhibitor Necrostatin-1 effectively hinders necroptosis by preventing the activation of RIPK1 (Xie *et al.*, 2013).

Necroptosis can additionally be triggered by Toll-like receptors (TLR4 and TLR3) and cytosolic nucleic acid sensors like RIG-I. This activation leads to the production of type I interferon (IFN-I) and TNF, thereby fostering necroptosis through an autocrine feedback loop (Brault *et al.*, 2018). TLR3 and TLR4 can also directly trigger necroptotic cell death through the interaction between TRIF and RIPK3 (Kaiser *et al.*, 2013). This leads to the activation of MLKL and the formation of a necrosome independently of RIPK1. Necroptosis induced by the activation of RIG-I and the adaptor molecule STING during virus infection involved the activation of RIPK1 through cIAP proteins (Schock *et al.*, 2017).

1.1.5 Pyroptosis

Pyroptosis is a type of cell death that is associated with inflammation, immune responses, and collateral damage. The term "pyroptosis," originally coined by Brennan and Cookson to describe a caspase-1-dependent lytic cell death, derives from the Greek words 'pyro,' signifying fire or fever, and 'ptosis,' indicating falling or dropping. This nomenclature serves to differentiate this process from apoptosis and necrosis. While it necessitates caspase 1, pyroptosis retains attributes associated with necrosis, which is a caspase-independent cell death (Brennan *et al.*, 2000).

Pyroptosis is typically associated with the activation of the inflammasome and Inflammatory caspases, which cleave gasdermin D (GSDMD) to form membrane pores, causing cell lysis and inflammation in the surrounding tissue (Zhao *et al.*, 2018). The Inflammasome serves as molecular platforms that trigger the induction of pro-inflammatory cytokines and the activation of inflammatory caspases. Depending on the specific type of inflammasome that is activated, pyroptosis is executed through two distinct pathways (Guo *et al.*, 2018b).

The induction of canonical pyroptosis or caspase 1-dependent pyroptosis relies on the activation of conventional inflammasomes. The majority of typical inflammasomes are assembled by pattern recognition receptors (PRRs) from nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) family, such as the NLR family pyrin domain-containing 3 (NLRP3), NLRP1 and NLRC4, and the adaptor molecule apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC). Nonetheless, Pyrin and the HIN200 protein AIM2 also form distinct and well-established inflammasomes (Guo *et al.*, 2015). In this canonical pathway, Pathogen-Associated Molecular Patterns (PAMPs) or damage-associated molecular pattern molecules

(DAMPs) trigger the activation of these inflammasomes, leading to the conversion of pro-caspase 1 into caspase 1. This caspase then cleaves pro-inflammatory cytokine precursors such as IL-1β and IL-18, as well as GSDMD. The N-terminal fragments of GSDMD, once cleaved, come together to form a transmembrane pore, rapidly causing cell membrane permeabilization and the release of cytoplasmic contents, including inflammatory cytokines (Xia *et al.*, 2021)

The non-canonical pyroptosis is initiated by non-conventional inflammasomes and caspase 4/5/11. In the noncanonical pathway, cytosolic sensing of lipopolysaccharides (LPS) directly activate caspase 4/5/11, which, in turn, cleave GSDMD and trigger pyroptotic cell death (Yang *et al.*, 2015).

Negative regulators of pyroptosis include protein kinase A (PKA), which inhibits the production of the N-terminal GSDMD fragment, and ESCRT-III, responsible for facilitating cell membrane repair upon GSDMD activation (Rühl *et al.*, 2018; Ye *et al.*, 2021).

1.1.6 Pyronecrosis

Pyronecrosis is a necrosis-like cell death or lytic cell death mode that relies on inflammasome adaptors. Although pyronecrosis is a term used to describe a type of cell death associated with pyroptosis, these two types of death have different characteristics. In contrast to pyroptosis, pyronecrosis occurs in a caspase-1-independent response to infection or inflammatory stimuli. Additionally, pyronecrosis can be distinguished based on its dependence on lysosomal cathepsins, such as cathepsin B (Averette *et al.*, 2009).

Pyronecrosis was first observed as a type of necrotic cell death induced in macrophages when they were infected with high proportions of the bacteria *Shigella flexneri*. The authors described that the stimulation of NLRs can trigger a type of necrotic cell death in the absence of caspase 1, which is why they concluded that it is a different type of death than pyroptosis, and called this type of death pyronecrosis. Its primary mode of operation involves NLRP3 receptor and the adaptor molecule ASC (Willingham *et al.*, 2007).

1.1.7 Lysosome-dependent cell death (LCD) and autophagy-dependent cell death (ADCD)

LCD is triggered by hydrolytic enzymes and cathepsins, potentially with the participation of MOMP and caspases (Wang *et al.*, 2018). LCD is characterized by the permeabilization of lysosomal membranes. This permeabilization of the lysosomal membrane triggers cell death signaling, particularly in the context of apoptosis, autophagy-related cell death, and ferroptosis. Lysosome-

released cathepsins have a crucial function in initiating cell death. In particular, cathepsin B has been demonstrated to activate proapoptotic proteins like BID and BAX (Repnik *et al.*, 2012).

ADCD is defined as a type of regulated cell death that fundamentally relies on the autophagic machinery or its components and is characterized by a distinctive morphological feature known as autophagic vacuolization. This feature can also be observed in the case of apoptotic or necrotic cell death (Denton *et al.*, 2019). Uncontrolled autophagy occurs during organ restructuring, when there is a lack of essential nutrients, or in specific pathological conditions (Yu *et al.*, 2018).

Autosis is a distinct form of cell death associated with ADCD, where the function of the plasma membrane Na^+/K^+-ATPase is of utmost importance. Autotic cells display extensive vacuolization, similar to the vacuoles seen in autophagy. These vacuoles are different from the classic autophagosomes and are a defining feature of autosis. Autotic cells exhibit specific morphological changes, such as increased cell volume and rounding of the cell, which distinguishes autosis from other forms of cell death (Liu *et al.*, 2015).

1.1.8 Ferroptosis

Ferroptosis characteristics have been identified in various organisms, including plants, and have been associated with a range of biological processes, spanning from development to aging, immune responses, and cancer (Dixon *et al.*, 2012).

Ferroptosis is strongly associated with lipid peroxidation and the depletion of cellular glutathione. Calcium has also been implicated in this form of RCD (Henke *et al.*, 2013). The iron-driven process that triggers lipid peroxidation during ferroptosis involves both non-enzymatic and enzymatic pathways. The peroxidation of cell membranes is facilitated by iron-containing lipoxygenases and free intracellular iron, instigating the generation of ROS through Fenton reactions. This lipid peroxidation primarily targets polyunsaturated fatty acids associated with phospholipids, including arachidonic acid (Yang *et al.*, 2016). Lipophilic radical traps such as vitamin E, ferrostatin-1, and lipoxstatin-1 can counteract the harmful consequences of lipid peroxidation during ferroptosis (Friedmann Angeli *et al.*, 2014).

1.1.9 PANoptosis

PANoptosis acts as a central hub for coordinating and regulating diverse cell death pathways within the immune response. PANoptosis has the ability to simultaneously regulate pyroptosis, apoptosis and necroptosis. This regulation occurs through the assembly of a multiprotein complex called the PANoptosome (Pandian *et al.*, 2022).

This complex comprises essential molecules that play roles in different forms of cell death. For pyroptosis, it involves caspase 1 and gasdermin D and E. For apoptosis, it includes caspase 8, caspase 3, and FADD. In the context of necroptosis, it features receptor-interacting protein kinase 3 (RIPK3) and MLKL (Wang *et al.*, 2021b).

AIM2, pyrin and the ZBP1-NLRP3 inflammasome are also part of this extensive protein complex (Lee *et al.*, 2021).

An additional pivotal controller of PANoptosis was revealed during an investigation involving the complex-I constituent, TAK1. The study unveiled TAK1's crucial involvement in NLRP3 inflammasome activation and its ability to modulate RIPK1 activity, thus governing PANoptosis (Malireddi *et al.*, 2019).

1.1.10 Immunological consequences of cell death

Cell death, especially when occurring in the context of infection or tissue damage, can have significant immunological consequences. The inflammatory response triggered by dying cells, whether in an acute or chronic context, plays a dual role by promoting tissue regeneration and infection control while potentially contributing to tissue injury and disease. Considering the pivotal role that inflammation plays in various human diseases, a thorough understanding of the fundamental mediators and pathways that orchestrate this response is crucial. The immunological consequences of cell death are diverse and depend on the specific type of cell death, the cellular context, and the molecules released during the process (Galluzzi *et al.*, 2017).

There are two types of immunological consequences of cell death. Tolerogenic cell death (TCD) is a phenomenon that triggers silent efferocytosis, in which dying or dead cells are peacefully eliminated by phagocytosis without provoking any inflammatory or immune reaction. In contrast, ICD activates the immune system, resulting in an immune response against antigens derived from the deceased cells (Green *et al.*, 2009a).

1.1.10.1 Tolerogenic cell death (TCD)

TCD is a form of cell death that actively suppresses immune responses, promoting a state of immunological tolerance. The term is often used to describe cell death processes that contribute to the maintenance of immune tolerance and prevention of excessive or inappropriate immune responses (Griffith *et al.*, 1996). The induction of tolerance by apoptotic cells depends on MHC class I molecules and leads to immune suppression of $CD8^+$ T cells, converting them into activated "helpless" cytotoxic $CD8^+$ T lymphocytes (CTLs) that function as primary effector T cells, but with a limited lifespan. These "helpless" CTLs can also undergo activation-induced cell death upon subsequent antigen exposure. This form of cell death is orchestrated by expression of the death ligand TRAIL, which induces apoptosis in both helpless CTLs and other activated T cells (Griffith *et al.*, 2007). The induction of TCD by dying cells can be influenced by receptors involved in the recognition and/or engulfment of apoptotic cells. The deletion or neutralization of certain molecules involved in engulfment, such as MER, MFG-E8, TIM4, or C1q, can compromise the induction of tolerance and lead to the induction of inflammation (Gaipl *et al.*, 2006).

A crucial mechanism in determining TCD involves the release of immunosuppressive cytokines, such as TGFβ, IL-10, prostaglandin E2, either from the dying cell itself or from the cell that engulfs it (Fadok *et al.*, 1998). Apoptotic cells can also stimulate the production of lipid mediators, including 15-lipoxygenase and 15-hydroxyeicosatetraenoic acid, which play a role in the resolution of inflammation (Serhan *et al.*, 2005). Moreover, dying cells can induce TCD by modifying DAMPs, such as HMGB1, during apoptosis. This neutralizes the ability of some DAMPS to induce ICD and promote tolerance (Kazama *et al.*, 2008).

1.1.10.2 Immunogenic cell death (ICD)

ICD is a unique type of RCD, characterized by its ability to induce an adaptive immune response targeting antigens from either the dying cell's own (endogenous) or external (pathogens) sources (Nagata *et al.*, 2017).

Initial understanding of the mechanisms of cell death suggested that the way a cell dies can influence the nature of the immune response it elicits. This concept arose from initial characterizations of apoptosis, described as a "silent death" with tolerogenic properties, in contrast to necrosis, characterized as a "violent death" that releases a variety of immunostimulatory molecules. Nevertheless, emerging evidence suggests that, under specific circumstances, stress-induced apoptosis can exhibit features of ICD, driving an inflammatory

response, culminating in the activation of CTL-driven adaptive immunity, along with the establishment of long-term immunologic memory (Galluzzi *et al.*, 2020).

ICD represents a functionally unique response pattern that comprises the induction of organellar and cellular stress, and culminates with cell death accompanied by the exposure, active secretion, or passive release of numerous DAMPs. For example, calreticulin, originating from the ER, translocates to the cell surface, serving as an "eat me" signal for phagocytes via CD91. This promotes the subsequent antigen cross-presentation to cytotoxic T cells and TH17 cell priming (Gardai *et al.*, 2005). ATP released from dying cells binds purinergic receptors on antigen-presenting cells, promoting recruitment, activation, and IL-1β secretion (Elliott *et al.*, 2009). dsDNA release from dying cells signals through the cytosolic DNA-sensing pathway cyclic GMP-AMP synthase (cGAS) and the adaptor molecule STING, amplifying a type I IFN response (Vanpouille-Box *et al.*, 2017). HMGB1 upon extracellular release, engages various PRRs including Toll-like receptor 2 (TLR2), TLR4, and RAGE, promoting cross-presentation and the induction of pro-inflammatory cytokines (Scaffidi *et al.*, 2002). The release of Annexin A1 from dying cells binds to Formyl Peptide Receptor 1 (FPR1) on phagocytes, which activates antigen processing and cross-presentation (Vacchelli *et al.*, 2015).

PART III : IMMUNE SENSING

The concept of immune sensing has been studied and described in various forms throughout the history of immunology research. A landmark paper that contributed significantly to our understanding of immune sensing is the publication by Charles Janeway Jr. (Janeway, 1989). He proposed the concept of "innate immunity" and discussed the role of PRRs in recognizing conserved molecular patterns on pathogens. He argued that the immune system possesses germline-encoded receptors capable of recognizing common microbial structures, which he referred to as PAMPs, resulting in an innate immune response. This paper laid the foundation for our understanding of immune sensing and the importance of innate immunity in host defense.

1.1 Innate immune sensing

Innate immune sensing enables the immune system to distinguish between the body’s own cells and potentially harmful pathogens or altered cells. This helps prevent autoimmune responses where the immune system attacks its own tissues (Lang *et al.*, 2007; Langan *et al.*, 2020; Singh, 2000). The immune system employs different mechanisms of immune sensing to identify

potential threats. One of the primary methods is via PRRs. They are proteins expressed by immune cells that can recognize specific molecular patterns commonly associated with pathogens, known as PAMPs (Table 1.1) (Calcagno *et al.*, 2022; Karnati *et al.*, 2015; Li *et al.*, 2021; Mohanty *et al.*, 2020; Tarigan *et al.*, 2020; Wang *et al.*, 2021a). The recognition of PAMPs by PRRs is a fundamental mechanism of the innate immune system, providing a rapid and generalized defense against a wide range of pathogens (Labarrere *et al.*, 2021).

Table 1.1 PAMPs and their source

Source of PAMPs	Bacterial	Viral	Fungal	Parasitic
	LPS found in the outer membrane of Gram-negative bacteria.	Double-stranded RNA (dsRNA), a replication intermediate in many RNA viruses.	β-glucans, a major component of fungal cell walls.	Glycosylphosphatidylinositol anchors present on the surface of some parasites.
	Peptidoglycan, a component of bacterial cell walls.	Single-stranded RNA (ssRNA) with specific features, such as certain secondary structures or sequences recognized by viral sensors.	Mannans, carbohydrate structures present in the cell wall of many fungi.	Specific parasite-derived proteins or nucleic acids.
	Flagellin, a protein component of bacterial flagella.	Viral proteins or nucleic acids not present in the host.	Chitin, a polysaccharide found in fungal cell walls and exoskeletons of insects.	
	Unmethylated CpG DNA motifs commonly found			

	in bacterial genomes.			

In addition to PAMPs, the immune system can also sense DAMPs. These molecules are released by damaged or stressed cells, indicating the presence of tissue injury.

The concept of DAMPs and their role in immune sensing and inflammation was first introduced by Polly Matzinger (Matzinger, 1994). She proposed the danger model of immune recognition, which challenged the traditional view of the immune system solely recognizing foreign antigens. Matzinger argued that the immune system is also attuned to detecting danger signals associated with tissue damage or cellular stress. While the specific term "DAMPs" was not used in this seminal paper, the underlying concept of DAMPs as danger signals which are released by damaged cells or tissues was discussed. Matzinger highlighted that molecules released or exposed by injured cells, such as heat shock proteins, could serve as danger signals that activate immune responses.

Examples of DAMPs are listed in Table 1.2. Similarly to PAMPs, DAMPs can activate PRRs and trigger immune responses (Gong *et al.*, 2020). Innate immune cells can also recognize abnormal or cancerous cells through the detection of DAMPs or alterations in cell surface markers. This early recognition can lead to the elimination of cancer cells or activation of adaptive immune responses against them (Lv *et al.*, 2020; Man *et al.*, 2022).

Table 1.2 DAMPs and their source

DAMPs	Source
High-Mobility Group Box 1 (HMGB1)	A nuclear protein that can be released by necrotic cells or actively secreted by immune cells during inflammation.
Heat Shock Proteins (HSPs)	Produced in response to cellular stress and can act as endogenous danger signals.
ATP	Released from damaged or dying cells and serves as a signal for inflammation.

DNA and RNA	Released from damaged or necrotic cells, these molecules can be recognized by the immune system as signs of cell injury.
Uric Acid	Produced during cell death and inflammation and can stimulate immune responses.
S100 Proteins	Calcium-binding proteins released by damaged cells.
Hydroxyapatite Crystals	Released from damaged bone tissue and can trigger inflammation.
Extracellular Matrix Components	Fragments of the extracellular matrix released during tissue injury can act as DAMPs.
Calreticulin	Released from dying cells from cancer therapy.
Mitochondria DAMPs (MTDs)	Trauma or Injury releases MTDs that contain genetic material (Mitochondria DNA (mtDNA)) and Mitochondrial components.
NETs	Released by neutrophils, NETs are fibrous structures of DNA histones, granular proteins, and cytoplasmic proteins.

Research on DAMPs and their role in immune sensing and inflammation has provided insights into tissue injury, repair processes, and the development of therapeutic interventions targeting DAMP signaling pathways (Anderton *et al.*, 2020; Roh *et al.*, 2018; Silvis *et al.*, 2020).

1.1.1 Pattern recognition receptors (PRRs)

PRRs are a class of proteins that are part of the immune system and are responsible for recognizing specific molecular patterns commonly found on pathogens or stressed cells (Isazadeh *et al.*, 2023; Janeway *et al.*, 2002). Although triggering of PRRs typically results in innate immune activation, it can also have inhibitory functions under specific circumstances (Fernández-García *et al.*, 2021). These are known as inhibitory PRRs. They are proposed to recognize patterns associated with both danger and homeostasis, providing a context for the

immune system to appropriately respond to potential threats, tolerate harmless substances, and maintain balanced immune responses (Rumpret *et al.*, 2022; Yu *et al.*, 2017).

There are several primary categories of PRRs that have been identified. These include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), scavenger receptors, cytosolic receptors, such as Nucleotide-Binding Oligomerization Domain (NOD)-like Receptors (NLRs), Retinoic Acid-Inducible Gene I (RIG-I)-like receptors (RLRs), cyclic GMP-AMP (cGAMP) synthase (cGAS), and a newly characterized class of DNA sensors, absent in melanoma 2 (AIM2)-like receptors (ALRs) (Li *et al.*, 2021; Vajjhala *et al.*, 2017; Wicherska-Pawłowska *et al.*, 2021).

The signaling pathways activated by PRRs are critical for initiating and coordinating immune responses. These pathways can vary depending on the type of PRR, the specific ligand it recognizes, and the cellular context (Cui *et al.*, 2014). In general, the signaling pathways activated by PRRs result in changes in gene expression. This includes the upregulation of genes encoding pro-inflammatory cytokines, chemokines, antimicrobial peptides, and other immune-related molecules. The production of pro-inflammatory cytokines and chemokines attracts immune cells to the site of infection or tissue damage. This amplifies the immune response and helps in the clearance of pathogens (Li *et al.*, 2021). PRR signaling can also lead to other cellular responses, such as phagocytosis, antigen presentation, and the induction apoptosis in infected or damaged cells (Rock *et al.*, 2011). It is noteworthy that different PRRs and their associated pathways can intersect and cross-regulate each other, providing a complex and finely tuned immune response tailored to the specific threat encountered.

1.1.1.1 Toll-like receptors (TLRs)

The concept of Toll-like receptors (TLRs) and their role in the immune system was significantly developed by Dr. Jules A. Hoffmann and Dr. Bruce A. Beutler (Beutler *et al.*, 2006; Lemaitre *et al.*, 1996). Their discoveries, which won the Nobel Prize in 2011,provided strong evidence for the existence of TLRs and their role in the immune response.

TLRs are evolutionarily conserved, indicating their importance in host defense. They are found in various species, from insects to mammals; in humans, there are 10 TLRs, while in mice, there are 13. These are present on the cell surface or within endosomes and recognize a wide range of PAMPs such as bacterial lipopolysaccharides, viral nucleic acids, and fungal cell wall components (Liu *et al.*, 2020; Rehli, 2002).

TLRs are expressed on various immune cells, including macrophages, dendritic cells, and B and T cells (Green *et al.*, 2009b; Maris *et al.*, 2006; Silva-Barrios *et al.*, 2016b; Xu *et al.*, 2017; Xu *et al.*, 2015). Certain TLRs are found on the cell surface, such as TLRs 1, 2, 4, 5, 6, while others are located in endosomes, such as TLRs 3, 7, 8, and 9. Different TLRs can recognize different PAMPs. For instance, TLR2 can detect bacterial lipoproteins, TLR3 detects double-stranded RNA, TLR4 detects bacterial LPS, TLR7 detects ssRNA and synthetic compounds—imidazoquinolines and loxoribine, and TLR9 can detect single-stranded DNA (ssDNA) containing CpG motifs (Table 1.3) (Lind *et al.*, 2022; Sameer *et al.*, 2021).

TLRs are designed to undergo a conformational change after ligand recognition, which initiates intracellular signaling cascades. This leads to the activation of transcription factors and the production of pro-inflammatory cytokines, chemokines, and IFN-I (Xu *et al.*, 2006). These molecules orchestrate the immune response, including the recruitment of other immune cells to the site of infection, antigen presentation to T cells, tissue repair, and maintenance of homeostasis after an infection or injury (Bonini *et al.*, 2005; Brint *et al.*, 2011; Rakoff-Nahoum *et al.*, 2009).

There are many majors signaling pathways associated with TLRs. For example, several TLRs are known to induce the NF-κB (nuclear factor kappa B) pathway. This pathway involves the activation and nuclear translocation of NF-κB. This transcription factor regulates the expression of genes involved in inflammation, immune responses, and cell survival (Gudowska-Sawczuk *et al.*, 2022). TLRs can also induce mitogen-activated protein kinase (MAPK) pathway, which includes ERK, JNK, and p38 (Brown *et al.*, 2011; Caruso *et al.*, 2014). These pathways are involved in regulating gene expression, cytokine production, and cell proliferation.

Another common pathway induced by TLRs is the Interferon Regulatory Factor (IRF) pathway. IRFs are transcription factors that play a key role in the regulation of immune responses, apoptosis, modulation of cell growth, differentiation, cellular metabolism, and oncogenesis. TLRs recruit adapter molecules to initiate this signaling pathway. The major adaptors involved in TLR signaling are MyD88 (myeloid differentiation primary response 88), TRIF (TIR-domain-containing adaptor protein inducing interferon-β), TRAM (TRIF-related adaptor molecule), and TIRAP (TIR domain-containing adaptor protein). These adaptors link TLRs to downstream signaling pathways (Akira *et al.*, 2004; Moynagh, 2005). The majority of TLRs signal through the MyD88-dependent pathway. After TLR activation, MyD88 is recruited to the TLR complex. This leads to the activation of IL-1 receptor-associated kinases (IRAKs) and TNFR-associated factor 6 (TRAF6), which in turn activate complex kinases to phosphorylates different transcription factors and allow them transcription factors to translocate into the nucleus. In the context of TLR7 signaling, MyD88 is

responsible for activating various downstream kinases and transcription factors, including IRF-5. MyD88-dependent activation of IRF-5 is a key step in the TLR7-mediated immune response (Schoenemeyer *et al.*, 2005). The function of IRF-5 and the role of TLR7 signaling in IRF-5 activation are detailed in a later chapter.

Some other TLRs, such as TLR3 and TLR4, can activate the TRIF-dependent pathway. TRIF is recruited to the TLR complex and initiates signaling through TANK-binding kinase 1 (TBK1) and IKKε. TLR signaling can also cross-regulate with other signaling pathways, including those initiated by cytokine receptors, to amplify immune responses (Takeuchi *et al.*, 2010).

Table 1.3 Human TLRs and their ligands

TLRs	Cell type / Localization	Ligand	Source
TLR1	Leukocytes **/** Plasma membrane	Triacylated lipopeptides, souble factors	Bacteria, mycobacteria *Neisseria emnngitidis*
TLR2	Monocytes, DC granulocytes, lymphocytes**/** Plasma membrane	Peptidoglycan (PGN), lipoprotein**/**lipopeptides, hepatitis c virus (HCV) core, heat shock proteins (HSPs)	Mycoplasma, non-lipopeptidic PAMPs from various pathogens,,HCV, host
TLR3	DC, NK, lymphocytes epithelial and endothelial cells**/** Endosome	Double-stranded RNA (dsRNA)	Viruses, bacteria, host
TLR4	Monocytes, DC, neutrophils, lymphocytes, epithelial cells **/** Plasma membrane	LPS, extracellular matrix components, HSPs, high-mobility group box 1 (HMGB1)	Gram-negative bacteria, host
TLR5	Leukocytes, epithelial cells **/** Plasma membrane	Flagellin	Bacterial flagella

TLR6	Leukocytes **/** Plasma membrane	Zymosan (ZMS), lipoteichoic acid (LTA), diacylated lipopeptides, soluble tuberculosis factor, outer surface protein A	Fungi, Gram-positive bacteria, mycoplasma, mycobacteria, *Staphylococcus*
TLR7	Leukocytes **/** Endosome	ssRNA, small interfering RNA (siRNA), imidazoquinolines (imiquimod (IMQ))	Viruses, bacteria, host, synthetic compounds
TLR8	Leukocytes **/** Endosome	ssRNA, siRNA, Human cardiac myosin, imidazoquinolines	Viruses, bacteria, host, synthetic compounds
TLR9	Leukocytes, microglial cells **/** Endosome	Unmethylated CpG containing DNA (CpG DNA)	Bacteria, virus, *Plasmodium,* host
TLR10	B cells plasmacytoid precursor DC **/** Endosome	Not determined	Not determined

1.1.1.2 Cytosolic receptors

Cytosolic receptors are a class of PRRs found inside the cell cytoplasm. Unlike cell surface receptors, cytosolic receptors are specialized in detecting intracellular threats, such as viral and bacterial components or damaged host cell molecules. These receptors play a critical role in initiating immune responses within the cell (Liwinski *et al.*, 2020). A list of known cytosolic receptors can be found in Table 1.4.

One example of cytosolic receptors are the RLRs. Their primary function is to detect and respond to various intracellular threats, including microbial pathogens and cellular stress signals. These receptors include RIG-I, Melanoma Differentiation-Associated Protein 5 (MDA5), and Laboratory of Genetics and Physiology 2 (LGP2), and recognize viral RNA, particularly double-stranded RNA (dsRNA) or 5'-triphosphate single-stranded RNA (5'-pppRNA) (Onoguchi *et al.*, 2011). When RLRs recognize viral RNA, they undergo a conformational change that allows them to interact

with an adaptor molecule called MAVS (Mitochondrial Antiviral Signaling protein) located on the outer mitochondrial membrane. Interaction between RLRs and MAVS leads to the activation of downstream signaling pathways, primarily the induction of IFN-I and pro-inflammatory cytokines, which is essential for initiating antiviral immune responses (Onomoto *et al.*, 2021; Rehwinkel *et al.*, 2020).

NLRs are another diverse family of cytosolic receptors that detect various intracellular threats, including bacterial or viral components and cellular stress signals, contributing to the regulation of inflammation and immune responses. NLRs are characterized by a tripartite domain structure. It is constituted by variable N-terminal domain, a central conserved nucleotide binding and oligomerization domain (NOD), which is central domain that responsible for nucleotide binding and oligomerization, and it is essential for signaling. The last part of this structure is a C-terminal domain, which is the domain that contains leucine-rich repeats and is involved in downstream signaling (Chen *et al.*, 2021). Examples of NLRs include NOD1 and NOD2, which specifically detect bacterial peptidoglycans, which are components of bacterial cell walls. NLRP3, NLRP1, and NLRC4 can respond to viral RNA and DNA, and can also sense cellular stress signals and DAMPs released during infection or tissue damage. These NLRs, can assemble into multiprotein complexes known as inflammasomes when they detect danger signals. Inflammasomes activate caspase-1, which leads to the processing and release of pro-inflammatory cytokines like interleukin-1β (IL-1β) and interleukin-18 (IL-18) (Ohto, 2022; Platnich *et al.*, 2019).

Another type of cytosolic receptor is cGAS that recognizes double-stranded DNA (dsDNA) present in the cytoplasm. cGAS detect both viral DNA and self-DNA that may be released or exposed during cellular stress or damage, such as that caused by genotoxic stress, chemotherapy, or certain types of cell death (Zhang *et al.*, 2014). The activation of cGAS catalyzes the production of cGAMP, which functions as a second messenger. cGAMP activates the stimulator of interferon genes (STING) pathway (Zhang *et al.*, 2013). Activated STING recruits the kinase TBK1 to the ER membrane, where STING is located. TBK1 phosphorylates NF-κB and Interferon Regulatory Factor 3 (IRF-3), which are held inactive in the cytoplasm in its unphosphorylated state. Phosphorylated IRF-3 or NF-κB then translocate into the cell nucleus, where they function as transcription factors. In the nucleus, they induce the expression of genes encoding IFN-I, which are important for antiviral immunity and immune regulation (Erttmann *et al.*, 2022; Kato *et al.*, 2013; Wu *et al.*, 2013b).

The most recently described cytosolic receptors family is ALR. They are a type of that specifically recognizes dsDNA. They are involved in detecting and responding to DNA-based danger signals,

such as DNA from pathogens or damaged host cells. They play a crucial role in initiating immune responses and maintaining cellular homeostasis (Gray *et al.*, 2016). ALRs, such as AIM2, Interferon Gamma Inducible Protein 16 (IFI16), and Myeloid cell Nuclear Differentiation Antigen (MNDA), can recognize dsDNA that is not properly sequestered within the cell. This DNA can be of microbial origin, or from damaged or stressed host cells. Some ALRs, especially AIM2 and IFI16, are involved in detecting viral DNA. ALRs are also implicated in responses to bacterial infections and inflammasome formation. AIM2 can assemble into a multiprotein complex called the AIM2 inflammasome when it binds to cytoplasmic dsDNA (Zhu *et al.*, 2019). Inflammasome activation leads to the cleavage of pro-caspase-1 into its active form, which subsequently processes pro-inflammatory cytokines (Caneparo *et al.*, 2018; Chen *et al.*, 2018; Wu *et al.*, 2013a).

Table 1.4 Cytosolic receptors and their functions

Cytosolic receptor family	Cytosolic receptor	Function
RLRs	RIG-I	RNA sensing. Respond to viral or host RNA, dsRNA, dsRNAs bearing 5′ triphosphate (5′ppp dsRNA) moiety or 5 diphosphate (5′pp dsRNA) end, circular RNA (circRNAs), ssRNA,
	MDA5	RNA sensing. Respond to viral or host long dsRNA, RNA (>1000 bp)
	LGP2	Regulation of RIG-I and MDA5
NLRs	CIITA (subgroup NLRA)	Regulation of MHC class II gene expression. CIITA is induced by IFNγ
	NAIP (subgroup NLRB)	Sensor of Flagellin, and inflammasome formation associated with NLRC4
	NOD1 (subgroup NLRC)	Recognition of D-γ-glutamyl-meso-DAP dipeptide (iE-DAP) or Diaminopimelic acid (DAP) (the motifs of PGN)
	NOD2 (subgroup NLRC)	Recognition of Muramyl dipeptide (MDP, the motif of PGN), viral ssRNA

	NLRC3 (subgroup NLRC)	Inhibition of T cell activation, TLR activation and STING signaling
	NLRC4 (subgroup NLRC)	Inflammasome receptors for flagellin, rod proteins. It is associated with NAIP
	NLRC5 (subgroup NLRC)	Regulation of MHC class I expression
	NLRX1 or NOD9 (subgroup NLRC)	Regulation of NF-κB and JNK signaling pathway, induction of reactive oxygen species (ROS) production in response to TNF or poly(I:C), modulation of autophagy induced by viral infection
	NLRP1 (subgroup NLRP)	Recognition of MDP, the motif of PGN, and anthrax toxin. Induction of inflammasome formation.
	NLRP2 (subgroup NLRP)	Negative regulation of NF-kB pathway and regulation of oogenesis, fertilisation and early embryonic development
	NLRP3 (subgroup NLRP)	Detection of different PAMPs, DAMPs. Induction of inflammasome formation by nucleic acids form invading pathogens or host, disruption of potassium homeostasis, lysosomal rupture, mitochondrial damage (mitochondrial ROS, mitochondrial DNA, or cardiolipin), mitotic factor NEK7, pore-forming toxins, crystalline substances (β-amyloid), Adenosine triphosphate (ATP) and hyaluronan.
	NLRP4 (subgroup NLRP)	Formation of inflammasome associated with NAIP, negative regulation of IFN I and modulation of autophagy in responses to virus or bacteria infection
	NLRP5 (subgroup NLRP)	Regulation of oogenesis, fertilisation and early embryonic development

	NLRP6 (subgroup NLRP)	Iduction of inflammasome formation, negative regulation of NF-kB pathway and modulation of self-renewal and integrity of the intestinal epithelium
	NLRP7 (subgroup NLRP)	Induction of inflammasome formation in response to microbial acylated lipopeptides. Regulation of trophoblast differentiation during early embryonic development through modulation of the BMP4 pathway
	NLRP8 (subgroup NLRP)	Not determined. Possible functions as inflammasome sensor in response to
	NLRP9 (subgroup NLRP)	Not determined. Possible functions in preimplantation embryo development and formation of inflammasome in response to virus (rotavirus) in the intestine
	NLRP10 (subgroup NLRP)	Migration of dendritic cells from inflamed tissue to the LN
	NLRP11 (subgroup NLRP)	Negative regulation of inflammatory signaling through repression of NLRP3 inflammasome, NF-κB pathway and type I interferon responses.
	NLRP12 (subgroup NLRP)	Part of the inflammasome and negative regulation of NF-kB pathway
	NLRP13 (subgroup NLRP)	Not determined. Possible role in inflammation
	NLRP14 (subgroup NLRP)	Modulation of spermatogenesis, fertilization, and inflammation
cGAS		Microbial dsDNA and self-DNA (damaged mtDNA, damaged nuclear DNA from chromosome instability, cytosolic DNA in micronuclei and from cell debris)

ALRs	AIM2	Sensor of microbial dsDNA and self-DNA. Induction of inflammasome formation.
	IFI16	Sensor of microbial dsDNA and self-DNA. Inhibition of inhibited activation of caspase-1 by the AIM2-ASC inflammasome.
	MNDA	Sensor of microbial dsDNA and self-DNA

1.1.1.3 C-type lectin receptors (CLRs)

CLRs are characterized by their ability to bind to carbohydrates, particularly mannose, fucose, and other sugar residues commonly found on the surface of microorganisms. This binding is mediated by specific carbohydrate recognition domains present in CLRs. Upon binding to their respective ligands on pathogens, CLRs can activate immune responses. These include the production of pro-inflammatory cytokines, chemokines, and the induction of phagocytosis. Moreover, CLRs on dendritic cells are involved in antigen presentation.

CLRs encompass a diverse family of receptors, each with distinct ligand specificities and functions. Examples of CLRs include Dendritic Cell-associated C-type Lectin-1 (Dectin-1) and -2 (Dectin-2), Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), CLR dendritic cell immunoreceptor (DCIR), and Macrophage-inducible C-type lectin. Dectin-1 is a type II transmembrane protein that it is also classified as class E scavenger receptors, because of its scavenger receptor activity. It functions as a PRR primarily expressed on the surface of immune cells like macrophages, dendritic cells, and neutrophils. Dectin-1 plays a critical role in recognizing and responding to fungal infections by detecting β-glucans, specific carbohydrates present on the surface of fungal cells. Activation of Dectin-1 initiates intracellular signaling cascades. This results in the production of pro-inflammatory cytokines, chemokines, and antimicrobial molecules, as well as the activation of phagocytosis. These responses are essential for the elimination of fungal pathogens (Drummond *et al.*, 2011). Furthermore, Dectin-1 signaling can synergize with Toll-like receptor (TLR) signaling to amplify immune responses against fungal infections. Emerging research suggests that Dectin-1 may also have roles in non-fungal contexts, including immune responses to certain bacteria and contributions to autoimmune diseases (Seeling *et al.*, 2023).

Others well known CLRs are DCIR and DC-SIGN that are primarily found on the surface of dendritic cells. These receptors can recognize glycosylated structures on viruses such as HIV. They have a CRD that can specifically bind to high-mannose glycans, a type of sugar structure with multiple mannose molecules attached, present on the envelope glycoprotein of HIV. Both receptors act as a receptor on dendritic cells that captures HIV particles (Cambi *et al.*, 2009; Lambert *et al.*, 2008). Once bound to these receptors, HIV can be internalized into the dendritic cells through endocytosis. In the lymph nodes, dendritic cells can interact with $CD4^+$ T cells, which are activated upon recognizing HIV antigens presented by the dendritic cells. This interaction initiates the adaptive immune responses, but it can also lead to the transmission of the virus from dendritic cells to $CD4^+$ T cells, facilitating HIV infection (Jin *et al.*, 2014). It is essential to emphasize that while these receptors facilitate the capture and transmission of HIV to $CD4^+$ T cells, it does not necessarily lead to HIV replication within dendritic cells. Instead, dendritic cells play a crucial role in initiating the immune response against HIV by presenting viral antigens to T cells (Su *et al.*, 2003).

1.1.1.4 Scavenger receptors (SRs)

The name "scavenger receptors" originates from their initial discovery as receptors involved in recognizing and clearing modified low-density lipoprotein particles, which are implicated in atherosclerosis. However, it is now understood that scavenger receptors have a much broader role beyond lipid metabolism (Goldstein *et al.*, 1979).

SRs are expressed by various immune cells, particularly myeloid cells. These receptors are designed to detect and bind a wide array of molecules, including both exogenous and endogenous ligands. (Alquraini *et al.*, 2020). SRs are required to eliminate apoptotic, impaired, or undesirable cells from the bloodstream (Terpstra *et al.*, 2000). This contribute to maintaining tissue homeostasis by helping to clear damaged or senescent cells, which is crucial for tissue repair and for preventing inflammation. SRs receptors can also interact with microbial ligands, promoting phagocytosis and activating immune responses against infections. For example, human monocyte scavenger receptors recognize the glucan structure from wall constituents of fungi and bacteria (Rice *et al.*, 2002). Another member of the scavenger receptor family, receptor for advanced glycation end products (RAGE), engages with PAMPs or DAMPs like advanced advanced glycation end products, HMGB1, and S100 proteins, thereby facilitating processes such as inflammation, oxidative stress, and apoptosis. (PrabhuDas *et al.*, 2017)

1.2 Immune sensing by cells of the adaptive immune system

Although PRRs are primarily associated with cells of the innate immune system, adaptive immune cells also express PRRs. However, T cells and B cells do not express the same array of PRRs as innate immune cells, and the consequences of PRR activation in lymphocytes differ based on factors such as the type of cell, its activation state, the specific ligand involved, and the microenvironment (Stögerer *et al.*, 2020). The PAMPs' recognition by PRRs is essential to initiate the signals required for the activation of lymphocytes, as well as their differentiation, leading to the production of effector cytokines (Bendelac *et al.*, 2002; Medzhitov *et al.*, 1997).

In B cells, PPRs-mediated sensing enhances B cell functions. For example, TLRs engagement can enhance the response initiated by BCR stimulation (Minguet *et al.*, 2008; Suthers *et al.*, 2017), and promote proliferation, differentiation, antigen presentation, and production of a range of cytokines and chemokines (Abu-Rish *et al.*, 2013; Agrawal *et al.*, 2011; Cognasse *et al.*, 2008; Jain *et al.*, 2013; Jiang *et al.*, 2007). Furthermore, TLRs can be implicate in BCR-complex reorganization, class switch recombination and maturation (Edry *et al.*, 2008; Hayashi *et al.*, 2005; Savage *et al.*, 2019). In contrast, PPRs-mediated sensing can have adverse effects in certain infections. For example, endosomal TLRs can activate B cells during *Leishmania donovani* and induce the production of IFN-I, which is detrimental for the host because it sustains hypergammaglobulinemia (Silva-Barrios *et al.*, 2016b).

Recently, it was demonstrated that recognition of molecular patterns, as opposed to specific epitopes recognized by BCRs, serves as a key factor in determining tolerogenic responses during peripheral B cell activation and this has been correlated with development of autoimmune diseases (Fillatreau *et al.*, 2021; Johnson *et al.*, 2019; Shen *et al.*, 2013).

Cytosolic DNA sensors are also important for B cell activation and function. Sensing the cytosolic DNA by B cells is fundamental for proper immune response during infection and cancer. It has been shown that cytosolic DNA and oligodeoxynucleotides (ODN) containing or not unmethylated CpG motifs (CpG DNA) induce activation, IgG class switch, and promote antigen presenting ability in B cells (Chen *et al.*, 2001; He *et al.*, 2004; Wang *et al.*, 2003). One of the important steps in the immune response to cytosolic DNA is the activation of cGAS-STING signaling. However, downstream signaling of STING has shown different and controversial functions. For example, activated STING initiates downstream signaling pathways that induce B cell activation and enhance antibody and cytokine production (Walker *et al.*, 2018). More recently, it has been shown

that STING signaling contributes to immunological tolerance in pancreatic cancer, inducing the expansion of human and mouse regulatory B cells (Li *et al.*, 2022). Conversely, STING is also implicated in tumor control in B cells since downstream STING signaling can induce apoptosis in malignant B cells. This is a key mechanism through which the body defends itself against abnormal cell growth and maintains tissue homeostasis. In addition, the ability to harness apoptosis for therapeutic purposes is a critical aspect of cancer research and treatment development (Tang *et al.*, 2016).

Similarly to B cell, T cells also express PPRs, including TLRs and cytosolic receptors (Hornung *et al.*, 2002). TLRs expressed on T cells can function as co-stimulatory receptors, but the expression and functional capacity of TLRs on $CD4^+$ and $CD8^+$ T cells is different (Rahman *et al.*, 2009). TLR2 expression is induced on $CD8^+$ T cells upon TCR activation, and this expression is higher than on $CD4^+$ T cells. TLR2 co-stimulation enhances TCR activation and plays a more significant role in promoting the proliferation and survival of $CD8^+$ T cells compared with $CD4^+$ T cells, based on differences in TLR2 expression levels between these T cell subsets (Lee *et al.*, 2009). Nonetheless, TLR2 plays an important role in $CD4^+$ T cells as well. TLR2 can serve as a co-stimulator during activation, it is also involved in the regulation of Th17 responses and maintenance of $CD4^+$ T cells memory (Komai-Koma *et al.*, 2004; Reynolds *et al.*, 2010). TLRs also play a crucial role in the in the expansion of effector and memory $CD4^+$ T cells. TLR9 activation increases the proliferation of effector $CD4^+$ T cells and makes these cells more resistant to the regulation exerted by regulatory T cells (Chiffoleau *et al.*, 2007). As well, the simultaneous activation of TLR5, TLR7, and TLR8 increases proliferation and cytokine production in memory $CD4^+$ T cells (Caron *et al.*, 2005). The cooperative action of different PRRs has also been involved in activation, survival, and proliferation of T cells. For example, TLR3 and TLR9 activation enhanced survival of $CD4^+$ T cells (Gelman *et al.*, 2004). As well, synergetic interaction of NOD1 and TLR2 amplifies the activation initiated by TCR in $CD8^+$ T cells (Mercier *et al.*, 2012; Pashenkov *et al.*, 2019). Other important PRRs for the proper functioning and survival of T cells are RLRs. For example, LGP2 enhances survival and expansion of antigen specific $CD8^+$ T cells in response to virus infection (Suthar *et al.*, 2012).

PPR activation can also inhibits TCR activation and regulates the T cell responses. For example, the presence of TLR4 on effector $CD4^+$ T cells is associated with an inhibitory role in regulating TCR activation and the inflammatory characteristics of T cells that contribute to colitis (González-Navajas *et al.*, 2010). In addition, Imanishi *et al.* showed mutual regulation between STING and TCR signaling pathways in T cells. Co-stimulation of T cells with cGAMP and TCR leads to IFN-I

production but inhibits T-cell proliferation, and this mutual regulation plays a role in modulating T-cell functions, including potential applications in antitumor responses (Imanishi *et al.*, 2019). Moreover, Uchimura *et al.* demonstrated that NLRC3 could limit Th1 and Th17 $CD4^+$ T cell activation, proliferation, and cytokine production. This study showed NLRC3 functions as a regulator of $CD4^+$ T cell activation when exposed to viruses, bacteria, or autoantigens, and this results support that PPRs can serves as a potential target for treating adaptive immune responses driven inflammation during chronic infections and autoimmune disorders (Uchimura *et al.*, 2018).

Despite all the of the studies that highlight how PRRs promote activation and survival of T cells, some PRRs have been associated with the induction of T cell anergy and cell death. For example, it has been demonstrated that when TLR7 is engaged in $CD4^+$ T cells, it leads to an intracellular calcium flux and activates an NFATc2-dependent program that induces T cell non-responsiveness, essentially rendering these T cells anergic. This study explored the implications of this TLR7-induced anergy in the context of HIV-1 infection. It is observed that silencing the TLR7 gene significantly reduces the frequency of HIV-1-infected $CD4^+$ T cells and restores their responsiveness. This discovery unveils a previously unknown role for microbial pattern recognition receptors in dampening immune responses, which has important implications for understanding immune dysfunction in chronic infections like HIV-1 (Dominguez-Villar *et al.*, 2015).

In addition, Fabié *et al.* showed that activation of TLR7 by apoptotic material promotes the expression and activation of Interferon Regulatory Factor 5 (IRF-5). Consequently, it induces death receptor 5 (DR5) expression and makes $CD4^+$ T cells more susceptible to cell death. The study suggests that in the presence of tissue disruption and chronic inflammation, commonly associated with persistent infections, the activation of TLR7 and IRF-5 in effector $CD4^+$ T cells may represent a shared pathway that suppresses protective $CD4^+$ T cells, ultimately favoring the establishment of chronic infection (Fabié *et al.*, 2018). Likewise, Larkin *et al.* indicate that STING agonists not only stimulate the production of IFN-I and the expression of IFN-stimulated genes, similar to the response seen in innate immune cells, but they can also activate pathways related to cellular stress and cell death in T cells. This suggests the need for a re-evaluation of therapies utilizing STING agonists to assess their potential effects on the T cell compartment (Larkin *et al.*, 2017).

In brief, PRRs in T and B cells expand the capabilities of the adaptive immune system by allowing these cells to directly sense pathogens and DAMPs, receive co-stimulatory signals, and fine-tune their responses. Understanding the roles of PRRs in these cells is crucial for unraveling the complexities of the immune response.

1.3 Type I interferon (IFN-I) and its role in immunity.

IFN-I are a group of signaling proteins consisting of 8 subtypes, interferon-alpha (IFN-α), interferon-beta (IFN-β), interferon-delta (IFN-δ), interferon-epsilon (IFN-ε), interferon-kappa (IFN-κ), interferon-omega (IFN-ω), interferon-tau (IFN-τ) and interferon-zeta (IFN-ζ). In human, IFN-I genes are situated at the position 9p21.3 and encode for IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω. IFN-δ, IFN-τ and IFN-ζ subtypes are not express in human, IFN-δ subtype is only express in non-primate and non-rodent placental mammals, IFN-τ is expressed in embryonic tissues during the early stages of gestation in sheep and cows, and IFN-ζ subtype is found in mice. Humans and many other species have multiple IFN-α subtypes, 13 distinct IFNα proteins are expressed in humans and 14 in mice. The others IFN-I classes are express as a unique protein functional form (Pestka *et al.*, 2004).

The production of IFN-I is initiated through the PAMPs' recognition by PRRs present in host cells. The activation of these PRRs triggers intracellular signaling cascades (Honda *et al.*, 2006a). Some of the key transcription factors activated during this process belongs to the IRF family, such as interferon regulatory factor 3 (IRF-3), IRF-5 or IRF-7; their activation depends on the cell type and the PRR involved. (Barnes *et al.*, 2001; Barro *et al.*, 2007).

Almost all cells in the body have the capacity to produce IFN-I (fibroblasts, epithelial cells, DC, monocytes, macrophages, Natural Killer (NK) cells, neutrophils, T and B cells). Plasmacytoid dendritic cells (pDCs) are particularly efficient at producing large amounts of IFN-I in response to viral infections. They express high levels of TLR7 and TLR9, which recognize viral nucleic acids and trigger IFN-I production (Bencze *et al.*, 2021; Zucchini *et al.*, 2008).

After translation, IFN-I proteins are secreted from the infected or stimulated cell into the extracellular space. IFN-I proteins bind to specific cell surface receptors on neighboring cells, known as IFN receptors (IFNAR). Binding of IFN-I to IFNAR triggers pathway and activates transcription factors like STAT1, STAT2 and IRF-5. These transcription factors induce the expression of hundreds of interferon-stimulated genes (ISGs) that play critical roles in antiviral defense and immune regulation. (Stewart *et al.*, 2001; Taniguchi *et al.*, 2001). Each type of IFN-I triggers the expression of a distinctive group of ISGs. Moreover, certain ISGs can be directly activated by viral infections, even in the absence of interferon production. This results in the activation of ISGs through a variety of interconnected mechanisms that frequently enhance one another (Crosse *et al.*, 2018).

IFN-I play a crucial role in the immune response, primarily by acting as signaling molecules that help the immune system combat viral infections and other pathogens. The products of ISGs contribute to various antiviral defenses, such as inhibiting viral replication, enhancing antigen presentation, and modulating immune responses (Honda *et al.*, 2005). IFN-I helps initiate and modulate both innate and adaptive immune responses. For instance, IFN-I activate dendritic cells, which are crucial for presenting antigens to T cells and initiating adaptive immunity (Le Bon *et al.*, 2002). Additionally, IFN-I contributes to the initiation of the inflammatory response, through the induction of proinflammatory cytokines and chemokines, which recruit immune cells to the site of infection or tissue damage (Goritzka *et al.*, 2014). IFN-I can also influence the differentiation and function of various immune cell types, including T cells, B cells, and regulatory T cells. This modulation helps tailor the immune response to specific threats (Gujer *et al.*, 2011; Le Bon *et al.*, 2003). Moreover, IFN-I enhance the activity of NK cells and cytotoxic T cells, which target and destroy virus-infected cells (Blackman *et al.*, 1985; Gibbert *et al.*, 2012).

While IFN-I is primarily known for its antiviral actions, it also plays a role in immune regulation. It can help maintain immune homeostasis by controlling the balance between pro-inflammatory and anti-inflammatory responses (Kumaran Satyanarayanan *et al.*, 2019). Some microRNA, short hairpin RNA (shRNA) and ISG function as a negative regulator of inflammatory immune response, such as activating signal cointegrator complex 3 (Ciechomska *et al.*, 2020; Li *et al.*, 2013). Moreover, IFN-I has antiproliferative and antitumor effects. It can inhibit the growth of cancer cells and promote their recognition and destruction by the immune system. This property has led to the use of IFN-I in cancer immunotherapy (Cauwels *et al.*, 2018; Takaoka *et al.*, 2003; Yanai *et al.*, 2002).

The role of IFN-Is has generally been considered beneficial against different infections and cancer. However, IFN-I signaling can also have a negative role in chronic virus infections and multiple cancer types (Snell *et al.*, 2017). Furthermore, IFN-I can induce immune dysfunctions through upregulating the expression of programmed death ligand 1, IL-10, and indoleamine 2,3 deoxygenase (IDO) on immune cells driving a suppressive response. that prevents the control of viruses and cancer (Cunningham *et al.*, 2016; Spranger *et al.*, 2013).

The majority of IFN-I pathways and ISGs target cellular proteins associated with innate defense mechanisms. However, it has recently been identified that ISGs are involved in cell cycle regulation, cellular metabolism, and apoptotic processes. This knowledge not only deepens our comprehension of the immune response but also opens up new avenues for research into potential therapeutic interventions and treatments related to immune-related diseases and

conditions, as well as cancer and metabolic disorders, where these pathways may be dysregulated (Hubel *et al.*, 2019).

PART IV: INTERFERON REGULATORY FACTOR 5 (IRF-5)

IRF-5 is part of the Interferon Regulatory Factors (IRFs) family. These transcription factors play critical roles in orchestrating immune responses. IRFs are involved in various physiological and pathological processes, regulating the expression of ISGs and initiating the immune response and the production of interferons (IFNs), which are important signaling proteins involved in defense against viral infections (Barnes *et al.*, 2002a). Among these, IRF-5 has emerged as a key transcription factor that plays a pivotal role in regulating the immune response, cellular metabolism, cell growth, differentiation, apoptosis, and modulating inflammatory pathways (Barnes *et al.*, 2004).

In humans, the *IRF-5* gene is situated on chromosome 7q32. This gene encodes a ~61-kDa protein (HuIRF-5), which was originally identified as a regulator of type I IFN gene expression (Barnes *et al.*, 2001). Due to high alternative splicing in the human *IRF*-5 gene, different IRF-5 splice variants or isoforms can be found. Certain of these variants have been recognized as genetic factors that increase the susceptibility to autoimmune diseases (Mancl *et al.*, 2005).

The *Irf-5* gene in mice is located on chromosome 6-12.36 cM. This gene, which encodes a ~ 55-kDa protein (MuIRF-5), is primarily expressed as a single predominant transcript of 498-amino acid. In C57BL/6J mice, a ~ 46-kDa minor spliced variant of MuIRF-5 with a 288-nucleotide deletion is observed exclusively in bone marrow and at low levels. MuIRF-5 exhibits approximately 87% amino acid sequence similarity to the full-length HuIRF-5, without internal deletions. The most notable contrast between human and mouse IRF-5 proteins is the high level of alternative splicing in the human IRF-5 gene that can result in different variants of this protein which can affect its functionality. MuIRF-5 has only one deletion of 5 amino acid and there is no evidence that this affects its function (Paun *et al.*, 2008). The mouse *Irf-5* gene, similarly to the human gene, consists of multiple exons (9 exons) that encode a transcription factor protein. It also contains various regulatory elements, including promoter regions that control its expression. The conservation of IRF-5 functions across species, including mice and humans, underscores its fundamental importance in immune responses (Eames *et al.*, 2016)

Studies of the three-dimensional structure of IRF-5 have provided insights into the specific folding and interactions of IRF-5 domains. The detailed structure of IRF-5 is complex and may vary in different conformations, depending on its activation state and interactions with other proteins, making its structure dynamic and context-dependent (Chen *et al.*, 2010). The structure of IRF-5 can be divided into several domains (Figure 1.2). Like other members of the IRF family, IRF-5 possesses a DNA binding domain (DBD) that allows it to specifically bind to DNA sequences known as interferon-stimulated response elements (ISRE) and is located in the N-terminal part. This domain is essential for its role in the regulation of genetic transcription. IRF-5 also contains an IRF-association domain (IAD), within the C-terminal activation domain (AD) that allows it to activate the transcription of target genes. This domain interacts with other proteins and transcriptional machinery to facilitate gene expression. Both domains, DBD and IAD domain, are connected by a linker region (LK), which is expected to adopt a folded conformation rather than remaining in an extended form. The activity of AD domain is regulated through various mechanisms, which involve alterations in conformation, either dependent or independent of phosphorylation events (Antonczyk *et al.*, 2019; Royer *et al.*, 2009).

For its activation, IRF-5 requires post-translational modifications and dimerization to be localized in the nucleus. IRF-5 also contains nuclear localization signals that facilitate its transport to the cell nucleus, where it can regulate gene transcription. Phosphorylation is a key post-translational modification that regulates IRF-5 activation and function. Phosphorylation of serine and threonine residues within the C-terminal autoinhibitory region acts as the trigger for initiating the activation of IRF-5 proteins within the cytoplasm. This activation process promotes dimerization, nuclear translocation, association with the coactivator CBP/p300, and the initiation of transcription. The specific phosphorylation sites in the C-terminal region are targeted by different kinases depending on the pathway that induces IRF-5 activation. Some of the well-characterized IRF-5 phosphorylation sites include Serine 425 (Ser425), Serine 427 (Ser427), Serine 430 (Ser430) Serine 436 (Ser436), Serine 462 (Ser462), Serine 158 (Ser158,) Serine 309 (Ser309), Serine 317 (Ser317), Serine 451 (Ser451) and threonine 10 (Thr10). Consequently, phosphorylation likely activates IRF-5 and other family members by instigating conformational changes that shift the role of the C-terminal segment from autoinhibitory to dimerization (Chang Foreman *et al.*, 2012; Chen *et al.*, 2008).

Table 1.5 Phosphorylation sites of IRF-5

Phosphorylation sites	Function
Ser425	Regulate dimer stabilization

Ser427	Release of autoinhibition
Ser430	Release of autoinhibition and nuclear retention
Ser436	Regulate dimer stabilization
Ser462	Regulate dimer stabilization and nuclear translocation
Ser158	Generally associated with transcriptional activation, but the precise mechanisms remain unknown
Ser309	Affect dimerization and contribute to the autoinhibitory release
Ser317	Generally associated with transcriptional activation, appear to play an auxiliary role
Ser451	Nuclear accumulation
Thr10	Generally associated with transcriptional activation, but the precise mechanisms remain unknown

Dimerization is important for its function as a transcription factor and its ability to bind to DNA. IRF-5 can form homodimers, where two IRF-5 molecules come together, or heterodimers, where IRF-5 interact with other transcription factors and co-activators. (Barnes, 2018; Chang Foreman *et al.*, 2012).

Not only phosphorylation events are important during IRF-5 activation, it has been shown that ubiquitination is also necessary for the correct activation of this transcription factor. Balkhi *et al.* reported that IRF-5 undergoes K63-linked ubiquitination catalyzed by TRAF6, a process that is important in facilitating IRF-5 nuclear translocation and subsequent regulation of target genes. Additionally, the necessity of IRAK1 for the ubiquitination of IRF-5 suggests that the phosphorylation of IRF-5 might be essential for facilitating effective ubiquitination (Balkhi *et al.*, 2008).

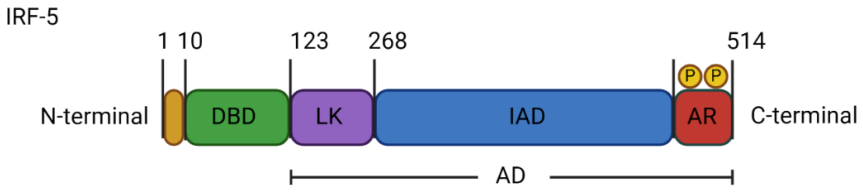

Figure 1.2 Functional domains of human IRF-5 protein

Schematic illustration of the functional domains of human IRF-5 protein. DNA binding domain (DBD) in green; linker region (LK) in purple; IRF association domain (IAD) in blue; autoinhibitory region (AR) in red: region of protein interaction, PEST and activation domain (AD) are indicated with a black line. Created with BioRender.com.

1.1 Isoforms

In the human genome, the *IRF-5* gene comprises a total of 9 coding exons, along with an additional noncoding exon located within the 5' untranslated region (UTR). Within this noncoding region, different promoters are situated upstream of the ATG start codon, which is found in exon 2. Four distinct variants (exon 1A, 1B, 1C and 1D) exist, each encoded by an alternative promoter for the *IRF-5* gene, designated as P-V1, P-V2, P-V3 and P-V4 (Clark *et al.*, 2013; Eames *et al.*, 2016).

The human *IRF-5* gene can undergo alternative splicing, resulting in the production of multiple isoforms. The exact number of isoforms may vary depending on the specific transcript variants and alternative splicing events considered. However, it is generally recognized that there are 11 isoforms (V1-V11) and at least four major isoforms of IRF-5 in humans (Figure 1.3) (Mancl *et al.*, 2005). These isoforms are commonly referred to as variant 1 (IRF-5V1), variant 2 (IRF-5V2), Variant 3 (IRF-5V3), and variant 4 (IRF-5V4). Each of these isoforms is initiated by one of four promoters, leading to transcripts that incorporate either exon 1A, 1B, 1C, or 1D resulting in variations in the composition of other exons. These isoforms exhibit specific expression patterns in different cell types, possess distinct subcellular localization characteristics, and serve unique functional roles in immune responses and disease pathogenesis. (Lin *et al.*, 2005; Mancl *et al.*, 2005). The functional properties of the IRF-5 isoforms can vary due to differences in their protein structure and composition resulting from alternative splicing events. Nevertheless, specific expression and functional details of each isoform are still under investigation, particularly in terms of their implication in autoimmune diseases (Kozyrev *et al.*, 2007; Stone *et al.*, 2013).

Thus far, 16 sequences of human *IRF*-5 have been submitted to NCBI GenBank™, but not all of these sequences have undergone functional characterization. There are specific IRF-5 isoforms that are unique and constitutively expressed, and they have the potential to influence the function of IRF-5 and the transcriptional patterns of genes regulated by IRF-5 (Graham *et al.*, 2006b). IRF-5V1 was the first published sequence in NCBI GenBank™ and it is the most studied and widely expressed IRF-5 isoform. This is the longest isoform of (~ 61 kDa) and it contains all the known functional domains of IRF-5. It is considered the canonical isoform and is involved in the transcriptional activation of pro-inflammatory cytokines (Cevik *et al.*, 2017; Wen *et al.*, 2011). It is known to be expressed in various immune cells, including unstimulated monocytes, macrophages, dendritic cells, and B cells and its expression increases in stimulated NK and T cells (Mancl *et al.*, 2005).

IRF-5V2 isoform is the shorter isoform, is generally expressed at lower levels than IRF-5V1 and can act as a negative regulator of IRF-5V1 function. (Lin *et al.*, 2005). IRF-5V2 is found in monocytes, NK cells and B cells, and at nearly undetectable in pDCs and T cells. (Mancl *et al.*, 2005).

IRF-5V3 and IRF-5V4 were identified for the first time in DCs. They are shorter isoforms with similar characteristics in their structure and function (Barnes *et al.*, 2001). It has been reported that IRF-5V3 and IRF-5V4 can be detected in unstimulated pDCs, monocytes, and B cells and it can be detected in NK and T cells after stimulation with Herpes simplex virus type I (HSV-1) (Mancl *et al.*, 2005). Additionally, it has been shown that these 2 isoforms enhance the transcriptional activity of IFNα gene promoters and triggers the expression of multiple IFNα genes in Newcastle disease virus (NDV)-infected cells (Barnes *et al.*, 2001). IRF-5V3 and IRF-5V4 also play a role in apoptosis and tumorigenicity, something that has been show as well for MuIRF-5 (Coccia *et al.*, 2004; Izaguirre *et al.*, 2003).

In addition to the initial four isoforms, 5 additional isoforms were identified. The variant 5 (IRF-5V5) and variant 6 (RF-5V6) were found in peripheral blood mononuclear cells (PBMCs) from healthy donors and in immortalized B and T human cancer cell lines (Mancl *et al.*, 2005).

The variant 7 (IRF-5V7), variant 8 (IRF-5V8) and variant 9 (IRF-5V9) were detected only in human cancer cell lines. These last 3 IRF-5 variants have the potential to act as dominant negative mutants, disrupting the activity of native IRF-5 (Eames *et al.*, 2016).

In 2006, Graham *et al* identified and cloned IRF-5V10 and IRF-5V11, which are previously undiscovered isoforms of IRF-5 from the mRNA found in the peripheral blood of individuals with SLE that were heterozygous for the rs2004640 genetic variant (Graham *et al.*, 2006c). This is a polymorphisms in the *IRF-5* gene that is associated with susceptibility to SLE (Sigurdsson *et al.*, 2005). This haplotype allows the expression of multiple IRF-5 isoforms containing exon 1B, a specific genetic variation. The presence of IRF-5 exon 1B isoforms, increased IRF-5 expression, and the risk of developing SLE.

Furthermore, an additional 14 *IRF-5* transcript variants originating from exon 1A were also identified (Stone *et al.*, 2013). These variants exhibit distinct expression patterns in both healthy individuals and patients with SLE. This underscores the intricate nature of IRF-5 expression and splicing and suggests the possibility of this gene serving as a biomarker for autoimmune diseases.

In 2014, Lazzari *et al*. found that after TLR7 stimulation distinct isoforms produced through alternative splicing are resistant to degradation. In this study, they demonstrated that the E3

ubiquitin ligase TRIpartite Motif 21 (TRIM21) acts as a suppressor of signaling pathways regulated by several members of the IRF family. TRIM21 control the function of these transcription factors by promoting ubiquitination-induced degradation. Analyzing the distinct IRF-5 variants reveals that alternative splicing isoforms are less prone to degradation mediated by TRIM21. This establishes a functional connection between the expression and activation of the isoforms with their stability. The lack of regulation of these isoforms could explain how IRF-5 promote a deregulated production of IFN-I and proinflammatory cytokines, which is essential in the development and pathogenesis of autoimmune diseases. (Lazzari *et al.*, 2014).

It is important to consider that the expression of IRF-5 isoforms can be context-dependent and the functional properties of each isoform may also depend on their interactions with other cellular factors, post-translational modifications, and cell type, immune stimuli, and disease conditions (Fu *et al.*, 2017).

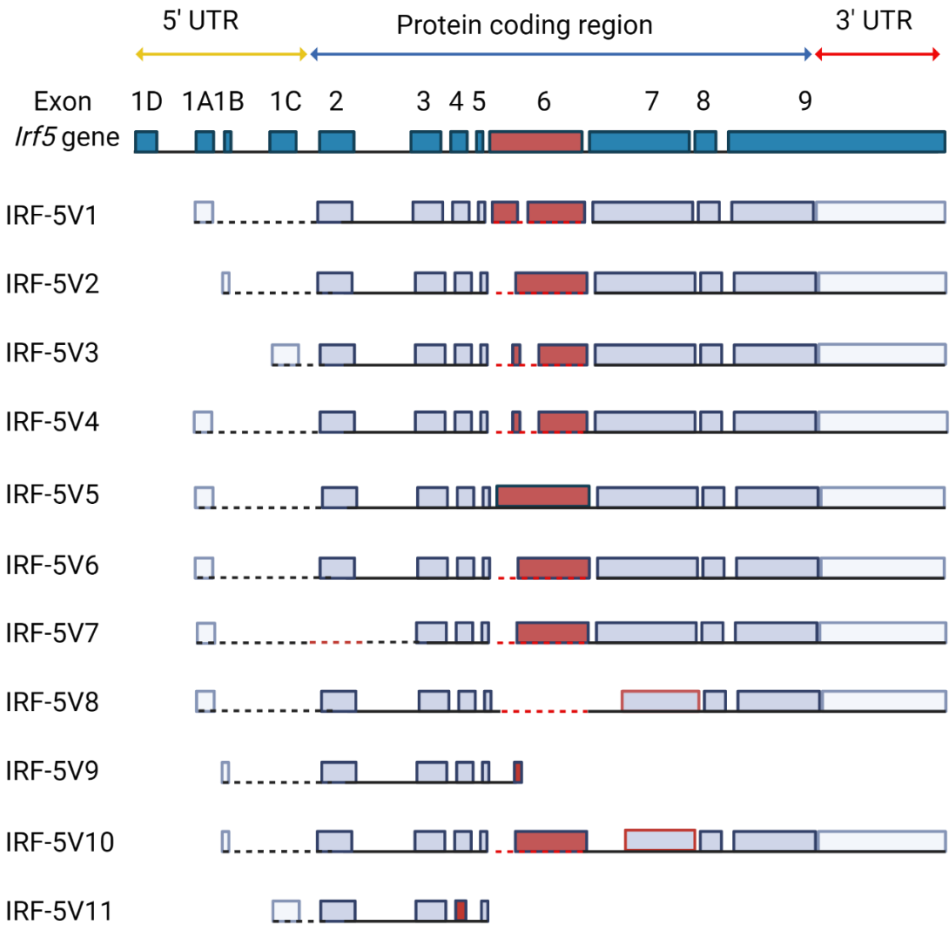

Figure 1.3 Principal human IRF-5 isoforms

Scheme illustrating the transcripts of the major human IRF-5 isoforms (IRF-5V1-V11). The illustration shows the different promoter exons (indicated by boxes in light blue) that each isoform uses and the insertion/deletion patterns (indicated by boxes and line in light red), specially in exon 6, present in some of the isoforms that result from alternative splicing events. Created with BioRender.com

1.2 Polymorphisms

It has been reported that functional Single Nucleotide Polymorphisms (SNPs) within the human *IRF*-5 gene can result in amino acid changes within the protein sequence. These variations can potentially affect the structure and function of the IRF-5 protein, leading to differences in its transcriptional activity, protein-protein interactions, or subcellular localization, and ultimately influencing the downstream immune signaling pathways regulated by IRF-5 (Rueda *et al.*, 2006). Certain IRF-5 polymorphisms have been associated with changes in the expression levels of IRF-5. Particularly, some polymorphisms can determine the expression of specific IRF-5 isoforms that contain insertions and deletions in the structure of IRF-5, which is associated with an imbalance in the immune response and the risk of autoimmune diseases (Cunninghame Graham *et al.*, 2007; Feng *et al.*, 2010b; Graham *et al.*, 2006b).

The exact number of known IRF-5 polymorphisms may vary as new research discoveries continue to emerge. Some of the commonly studied IRF-5 polymorphisms have been associated with various autoimmune (Peng *et al.*, 2021; Rueda *et al.*, 2006; Sigurdsson *et al.*, 2008). These polymorphisms contribute to the dysregulation of immune responses and increase the likelihood of developing inflammatory and autoimmune conditions. The specific functional mechanisms underlying disease susceptibility may involve altered gene expression, disrupted protein interactions, or aberrant immune signaling pathways (Han *et al.*, 2009).

One of the most studied IRF-5 polymorphisms is rs2004640, also called risk-associated T allele. The association of this IRF-5 polymorphism with inflammatory diseases and autoimmune diseases may vary among different populations and ethnic groups. (Bae *et al.*, 2019; Kawasaki *et al.*, 2008). (Devaraju *et al.*, 2018; Rueda *et al.*, 2006).

Another well studied polymorphism is rs77571059; an insertion/deletion of a 5-base pair indel situated 64 base pairs upstream of the alternate exon 1A within the IRF-5 gene. This SNP has been linked to an increased susceptibility to SLE, inflammatory bowel diseases, multiple sclerosis, Sjögren's syndrome and acute coronary syndrome (Dideberg *et al.*, 2007; Fan *et al.*, 2010; Kristjansdottir *et al.*, 2008; Sigurdsson *et al.*, 2005).

Other studies have explored genetic links between IRF-5 polymorphisms and genes close to IRF-5, such as Transportin-3 (TNPO3), that also contain lupus-associated variants (Kottyan *et al.*, 2015). The TNPO3 gene encodes for a nuclear import receptor involved in nucleocytoplasmic transport of proteins. TNPO3 protein is additionally implicated in HIV-1 infection, due to its interaction with the HIV-1 capsid protein (Diaz-Griffero, 2012).
Kottyan *et al*. identified rs4728142, a variant linked to the IRF-5 promoter, enhances the binding of the transcription factor ZBTB3 and increase the expression of the IRF-5 gene (Kottyan *et al.*, 2015).

1.3 Regulation of IRF-5 expression

As previously discussed, IRF-5 expression levels depend on the type of isoform, the cell type, its regulation, and the presence of polymorphisms.

One of the mechanisms that determines the expression level of some of the known variants of the IRF-5 transcript is polyadenylation. Some polymorphisms create functional polyadenylation sites that increase the expression of specific transcript variants. This type of polymorphism is a determining factor in the expression of the IRF-5 gene and its correlation with SLE (Cunninghame Graham *et al.*, 2007).

The functionality of the IRF-5 is determined by its activation state. Alteration of one of the essential events for activation of IRF-5 (phosphorylation, ubiquitination, dimerization) or impediment in its translocation to the cell nucleus, pairing with cofactors or binding to gene promoters negatively regulates the functioning of IRF-5. The functionality of IRF-5 is influenced by the specific type of dimerization and the proteins with which it forms these dimers. The formation of the IRF-5/IRF-7 heterodimer does not have a cooperative effect; instead, it leads to a repression of *IFNA* gene transcription rather than enhancement. The interaction between IRF-5 and IRF-7 relies on the phosphorylation of IRF-7. These heterodimers are formed through the amino terminus, which masks the DNA binding domain. Consequently, this alteration affects the composition of the enhanceosome complex binding to *IFNA* promoters and modulates the expression of various *IFNA* subtypes. These findings suggest that IRF-5 can function both as an activator and a repressor of IFN gene expression, depending on its interaction partner (Barnes *et al.*, 2003a).

In general, the translocation of transcription factors to the nucleus is a crucial process for their proper functioning. The subcellular localization of IRF-5 is regulated through importins and

exportins that transport IRF-5 between the nucleus and cytoplasm. IRF-5 predominantly resides in the cytoplasm in its unstimulated state, primarily due to the influence of its functional nuclear export signal (NES). One particular export receptor, Chromosomal Maintenance 1 (CRM1), plays a significant role in this process. CRM1 interact with IRF-5 after dephosphorylation in the nucleus. CRM1 directly recognizes sequences containing NES and export IRF-5 to the cytoplasm (Lin *et al.*, 2005). Conversely, IRF-5 active form is transported into the nucleus with the aid of the carrier proteins karyopherin (KAP)-α1 and -β1. These proteins serve as transport receptors, functioning to escort cargo proteins into the nucleus. (Yeon *et al.*, 2008). Mutations in NES affect the intricate transport system that ensures precise control over IRF-5 functionality and can result in the continuous presence of IRF-5 in the nucleus (Lin *et al.*, 2005).

Another way to regulate IRF-5 expression and function is through epigenetic mechanisms. Some studies have indicated that hypermethylation of promoter CpG island associated with inactivation of *irf-5* gene expression (Dong *et al.*, 2015; Shin *et al.*, 2010). Regulation of IRF-5 function is also controlled through an interaction with histone acetyltransferases (HAT) and histone deacetylases (HDACs). This interaction, with both HDACs and HATs, leads to changes in IRF-5 ability to activate gene expression. Specifically, HDACs are shown to be required for the transactivation and transcription of certain gene promoters (*ISRE, IFNA*, and *IL6*), while not being necessary for others (*TNF*). HAT interacts with phosphorylated IRF-5, leading to IRF-5 acetylation and inhibiting the recruitment of IRF-5 to *IFNA* promoters. HATs also form a complex with the corepressor proteins SMRT (silencing mediator for retinoid or thyroid-hormone receptor) and Sin3a that associate with IRF-5 can block *IFNA* gene expression (Feng *et al.*, 2010a).

Moreover, the expression of IRF-5 can be post-transcriptionally modulated by microRNAs, which can inhibit translation of IRF-5 mRNA into protein and affecting the expression of different IRF-5 target genes (Chang *et al.*, 2021; Chen *et al.*, 2017; Fang *et al.*, 2021; Gong *et al.*, 2018; Lin *et al.*, 2022; Lv *et al.*, 2021a; Tang *et al.*, 2009; Wang *et al.*, 2021c).

1.4 IRF-5 function

IRF-5 is primarily expressed in leukocytes, but it can be found in non-immune cells, such as fibroblasts, endothelial cells, and chondrocytes.

In fibroblasts, IRF-5 serves as a tumor suppression. Indeed, *Irf5*$^{-/-}$ fibroblasts display resistance to apoptosis revealing its critical function in this process, particularly in response to DNA damage (Yanai *et al.*, 2007). IRF-5 is a critical regulator of endothelial inflammation. In human endothelial cells, this transcription factor upregulates the expression of vascular cell adhesion molecule 1

(VCAM-1) via Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway during ischemia-reperfusion injury. This regulates the attachment of leukocytes to endothelial cells, contributing to the pathological process in IRI (Cai *et al.*, 2017). IRF-5 also plays a significant role in the regulation of Matrix Metalloproteinase-3 (MMP-3) expression in chondrocytes, which are crucial cells in the context of osteoarthritis. MMP-3 is involved in the degradation of articular cartilage in OA. (Guo *et al.*, 2018a).

Nevertheless, IRF-5 is mostly known for its involvement in the regulation of immune and inflammatory genes. The disruption of IRF-5 function has been linked to the development of numerous autoimmune and inflammatory disorders, including systemic lupus erythematosus (SLE), rheumatoid arthritis, Sjögren's syndrome, and inflammatory bowel disease. Genetic variations in the IRF-5 gene have been associated with an increased risk of developing these conditions, highlighting the importance of IRF-5 in immune dysregulation (Dideberg *et al.*, 2007; Dieguez-Gonzalez *et al.*, 2008; Miceli-Richard *et al.*, 2007).

In leukocytes, IRF-5 is expressed at different levels and has different functions in each cell type, depending on the stimuli that induce its activation. IRF-5 is expressed more highly in monocytes and macrophages, less in dendritic cells and B cells, and it is found at very low levels in T cells and NK cells (Li *et al.*, 2016a). The activation of IRF-5 involves different signal transduction pathways, including the TLR, RIG-I-like RNA helicase, Dectin-1, NOD2, NF-κB, JAK-STAT, Fas, TNF-related apoptosis-inducing ligand (TRAIL), and IFN-I-mediated signaling pathways. These pathways use different adapter proteins and kinases for the activation of IRF-5 (Figure 1.4) (Ryzhakov *et al.*, 2015).

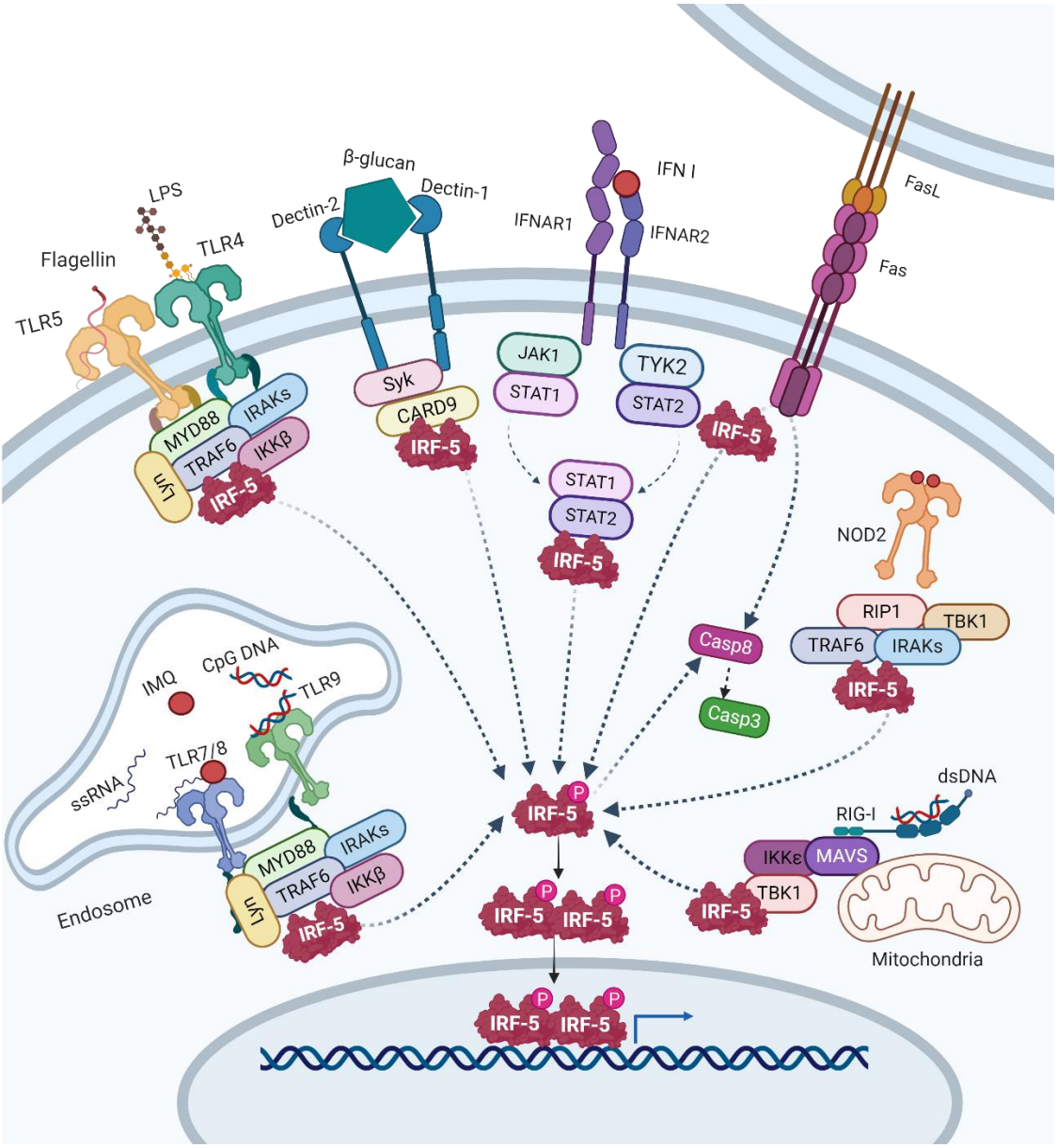

Figure 1.4 Activation of IRF-5 by different signal transduction pathways in leukocytes.

IRF-5 is upregulated upon activation of several TLRs, such as TLR4, TLR5, TLR7, TLR8 and TLR9 in leukocytes. In APCs, IRF-5 is induced by *Candida albicans*. It is dependent on Dectin-1- and Dectin-2-mediated signaling to produce IFN-β. In addition, IRF-5 is strongly induced by type I IFN-mediated signaling. The binding of IFN-I to the receptor results in the activation of IRF-5. Cytosolic receptors such as RIG-I and NOD2 can also induce IRF-5. Alternatively, Fas stimulation activates IRF-5 in response to DNA damage. Created with BioRender.com.

1.4.1 The role IRF-5 in antigen presenting cells

IRF-5 was characterized for the first time in DCs, where it was found to stimulate *IFNA* gene expression, even in the absence of IRF-7. Notably, IRF-5 displays a unique response pattern in that it promotes *IFNA* gene induction specifically in NDV-infected cells, with distinct expression profiles. Indeed, IRF-7 predominantly induces *IFNA1*, while IRF-5 primarily induces *IFNA8* (Barnes et al., 2001).

However, the activation of inflammatory genes in infected cells by IRF-5 extends beyond just the type I interferon genes. IRF-5 can promote the expression of cytokines such as IL-6, TNF, and IL-12. (Krausgruber *et al.*, 2010; Takaoka *et al.*, 2005). In addition, IRF-5 can induce chemokines, such as RANTES (also known as CCL5), monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α) and 1β (MIP-1β), interleukin-8 (IL-8), and CXC motif chemokine 10 (CXCL10, also known as interferon γ-induced protein 10 kDa (IP-10)) (Barnes *et al.*, 2002b).

It has recently been demonstrated that IRF-5 plays a regulatory role in controlling a new specific set of genes in dendritic cells during virus infection. A study showed that there is a remarkable change towards genes from immune-related pathways, including the dendritic cells maturation, IL-1 mediation of RXR, and NFAT regulation of immune responses after West Nile virus (WNV) infection. These datasets uncover new binding motifs for IRF-5 beyond the well-established canonical IRF binding motif. They also shed light on gene loci that were previously unknown to be under the regulation of IRF-5. This research showed the unique and specific program of innate immunity and immune gene regulation orchestrated by IRF-5 (Chow *et al.*, 2019).

IRF-5 also modulates DCs' maturation by cooperating with IRF-1, IRF-2, and NF-κB (p50, p65, and cRel) family members to regulate the expression of maturation-specific markers CD83. CD83 is upregulated in fully mature DCs, and its regulation depends on chromatin rearrangement, transcriptional regulatory elements and molecular machinery, including activators and transcription factors and DNA binding sites for activators, called transcription factor binding sites. IRF-5, IRF-1, IRF-2 and NF-κB family members form a complex within these regions and have a synergistic effect in regulating human CD83 expression (Stein *et al.*, 2013).

IRF-5 is also known to be a specific marker of inflammatory monocytes and macrophages. It has been shown to promote the differentiation of monocytes into pro-inflammatory M1 macrophages (Krausgruber *et al.*, 2011; Weiss *et al.*, 2013) and to directly initiate the transcription of genes responsible for IL-12 subunit p40 (IL-12p40), IL-12 subunit p35 (IL-12p35), and IL-12 subunit p19 (IL-12p19), while suppressing the gene for IL-10 (Krausgruber *et al.*, 2011).

IRF-5 is also enhancing bacterial clearance in PRR-stimulated, M1-differentiated human macrophages. The pathway involves the activation of various mechanisms, including the induction of reactive oxygen species, nitric oxide synthase, and autophagy. These pathways are activated through IRF-5-dependent MAPK, NF-κB, and Akt2 signaling. Nevertheless, variations in the IRF-5 gene associated with immune-mediated disease risk can influence these antimicrobial pathways, and potentially affecting an individual's susceptibility to microbial-related diseases (Hedl *et al.*, 2019).

In mice, IRF-5 also regulates the mononuclear phagocytes system within the colon and serves as a pivotal factor in initiating intestinal inflammation. IRF-5 facilitates the differentiation of monocytes into $CD11c^+$ macrophages with pro-inflammatory properties and regulates the generation of antimicrobial and inflammatory mediators by this cell population (Corbin *et al.*, 2020). These inflammatory $CD11c^+F4/80^+$ macrophages promote intestinal inflammation and control the progression of immunopathological response during *Helicobacter hepaticus* induced colitis, contributing to immunopathology by generating pro-inflammatory cytokines like IL-23, IL-1β, and TNF. Pandey *et al.* showed that mice deficient in IRF-5 have less severe colitis but were less capable of effectively eliminating intestinal pathogens. This study identified that IRF-5 in macrophages was crucial for phagocytosis and for activating NFκB-dependent pathways involved in clearing intracellular bacteria. However, even though bacterial clearance pathways were reduced, mice with IRF-5 deficiency had lower inflammatory cytokine levels, which led to reduced intestinal permeability, bacterial translocation, and intestinal inflammation (Pandey *et al.*, 2019). Interestingly, this $CD11c^+$ macrophage phenotype induced by IRF-5 is not specific for inflammatory diseases in the intestine. These macrophages are also involved in atherosclerotic plaque inflammation and carotid plaque rupture in humans and mice (Edsfeldt *et al.*, 2022).

In addition, IRF-5 expression in myeloid cells is crucial for inducing splenomegaly and recruiting myeloid cells to the spleen during chronic visceral leishmaniasis (VL) (Mai *et al.*, 2019). During *L. donovani* infection, IRF-5 also induces IL-12 production and modulates HIF-1α expression in DCs. The expression of HIF-1α in $CD11c^+$ cells promotes inflammation, affects $CD8^+$ T cell responses, increases splenic parasite load and amplifies the disease (Hammami *et al.*, 2017; Hammami *et al.*, 2015; Paun *et al.*, 2011).

1.4.2 Signaling pathways in APCs

Various pathways leading to IRF-5 activation have been identified in APCs. One of the most extensively studied mechanisms is the activation of IRF-5 through the TLR pathway. The study

by Takaoka *et al.* marked the pioneering observation that the presence of IRF-5 is indispensable for the TLR-driven initiation of genes associated with pro-inflammatory cytokines. IRF-5 activation is limited to TLRs that transmit signals through the MyD88, such as TLR4, TLR5, TLR7, and TLR9. This adaptor protein forms a complex that involves IRAK1, IRAK4 and TRAF6. The IRAKs activate TRAF6, which then mediated the ubiquitination of IRF-5. TRAF6 also activates the downstream kinase transforming growth factor β-activated kinase 1 (TAK1) and IκB kinase β (IKKβ). Finally, the kinase IRAK4, operating through TAK1 and IKKβ, triggers RF-5 phosphorylation for its activation (Takaoka *et al.*, 2005). The majority of TLRs that employ the Myd88-dependent pathway also activate NF-kB. This results in the phosphorylation of inhibitory IkB proteins, allowing NF-kB to enter the nucleus, where it collaborates with IRF-5 to induce the expression of proinflammatory genes (Moynagh, 2005).

Furthermore, IRF-5 can promote TNF induction in $CD11c^+$ DCs through TLR3, TLR4, and TLR9 signaling pathways. On the other hand, IRF-5 is specifically required for TLR9-mediated type I IFN transcription in these cells, distinct from its role in TLR3 and TLR4 signaling (Paun *et al.*, 2008).

Schoenemeyer and collaborators described for the first time that TLR7 was upstream of IRF-5. In this study, IRF-5 expression was dependent on MyD88, IRAK1 and TRAF6 following TLR7 stimulation, in contrast to IRF3, which is regulated by TRIF in the TLR3 or TLR4 signaling pathway (Schoenemeyer *et al.*, 2005). The importance of this pathway has been identified in the context of infectious diseases. For example, myeloid DCs (mDCs) in SARS-CoV-2-infected patients exhibited lower levels of the transcription factor IRF-5 at baseline. Furthermore, mDCs and pDCs from these patients tended to upregulate IRF-5 less than healthy individuals when stimulated with TLR7 agonists. DCs from SARS-CoV-2-infected patients produced lower IL-6 after *ex vivo* TLR7 stimulation and the frequency mDCs positive for IRF-5 correlated with mDCs producing IL-6 and TNF (Cords *et al.*, 2023).

In human primary phagocytes, TLR8 can detect *Staphylococcus aureus,* activate IRF-5, and induce the production of IFN-β and IL-12. TLR8 activates IRF-5 through a signaling pathway involving TAK1 and IKKβ. However, TLR2 signaling can antagonize this pathway (Bergstrøm *et al.*, 2015). In the case of TLR9, the induction of the expression of various genes associated with immune responses, including CXCL2 and IκBζ, is also contingent on IRF-5 (Negishi *et al.*, 2005).

Other proteins important for the activation of IRF-5 through endosomal TLRs are endolysosomal transporter SLC15A4 and TLR adapter that interacts with SLC15A4 in the lysosome. Heinz *et al.* recently reported that the interaction of TASL with SLC15A4 is crucial for TLR7, TLR8 and

particularly TLR9 signaling in the activation of the IRF-5 pathway. The discovery of a conserved functional pLxIS motif (where p denotes a hydrophilic residue and x denotes any residue) in TASL, which mediates the recruitment and activation of IRF-5, revealed its function as an essential innate immune adapter required to recruit and activate IRF-5 through TLR7-9. This discovery highlights a striking mechanistic similarity to IRF-3 and its three adaptors, STING, MAVS, and TRIF (Heinz *et al.*, 2020).

The IRF-5 activation by TLR pathway can be regulate through the inhibition of transcriptional modifications. For example, Lyn, a member of the Src family kinase can prevent the transcriptional modifications of IRF-5 induced by the TLR-MyD88 pathway (Ban *et al.*, 2016). Moreover, IκB Kinase α (IKKα) can phosphorylate IRF-5, resulting in the formation of IRF-5 dimers, which typically indicate activation. However, this phosphorylation by IKKα actually inhibits IRF-5's ability to activate the transcription of IFN-I and inflammatory cytokine. TLR-MyD88 pathway also induces the expression of alkaline phosphatase, which leads to IRF-5 de-phosphorylation, controlling the inflammatory response by decreasing IRF-5 activity (Balkhi *et al.*, 2010).

In addition, other members of the IRF family serve as a negative regulator of TLR-IRF-5 signaling. For instance, IRF-4 inhibits the IRF-5 activation through competition with IRF-5 for interaction with MyD88. (Negishi *et al.*, 2005).

Conversely, it has been shown that some proteins, such as the Nuclear RNA export factor 1 (NXF1), act as a positive regulator of TLR7-IRF-5 signaling pathway. Initially, NXF1 was characterized as an RNA-binding protein responsible for transporting processed mRNA out of the nucleus, but NXF1 is also found in complexes with IRF-5, and their interactions are strengthened when TLR7 is activated. (Fu *et al.*, 2017). RelA is another protein that amplifies the TLR-IRF-5 signaling pathway. RelA facilitates the recruitment of IRF-5 to the promoter of inflammatory genes after stimulation of TLR4 with LPS (Saliba *et al.*, 2014).

Besides TLRs, other signal transduction pathways promoting IRF-5 activation in APCs include the Dectin-1, the NOD2, RIGI and MDA5, the protein tyrosine kinase 2-beta, and the Fas/FasL pathway. The Dectin-1 signaling leads to IRF-5 activation, which promotes IFN-β production in response to fungal infection caused by *Candida albicans* in DCs. The activation of IRF-5 by the C-type lectin receptor Dectin-1 depends on the participation of the tyrosine kinase Syk and the adapter protein Card9 (del Fresno *et al.*, 2013).

Cytosolic recognition of both bacteria and viruses through NOD2 also leads to IRF-5 activation. For example, *Mycobacterium tuberculosis* activates the NOD2 pathway, which leads to the expression of IFN-I via IRF-5 in a way that depends on the proteins RIP2 and TBK1 (Pandey *et*

al., 2009). Human macrophages, stimulation of NOD2 induces the activation of IRF-5 and its association with RIP2, IRAK1 and TRAF6. This interaction is essential for optimal Akt2 activation, resulting in elevated expression of glycolytic pathway genes, HIF1A, pro-inflammatory cytokines, and M1 polarization (Hedl *et al.*, 2016).

The interaction of RLRs (RIG-I and MDA5) with the mitochondria-associated adapter molecule MAVS also promotes the activation of IRF-5 through the kinases TBK1 and IKK-ε. Lazear *et al.* demonstrated that the activation of interferon-stimulated genes (ISGs) via MAVS can occur through a pathway dependent on IRF-5, even in the absence of IRF-3 and IRF-7 in mDCs during WNV infection (Lazear *et al.*, 2013).

Not long ago, protein tyrosine kinase 2-beta (PYK2) was reported to be a novel protein that plays a crucial role in macrophage-mediated IRF-5 activation and subsequent inflammatory reactions within the gastrointestinal tract. The study identifies PYK2 as a kinase responsible for activating IRF-5 and demonstrates that PYK2-deficient macrophages have reduced IRF-5 activation, leading to decreased expression of inflammatory genes. (Ryzhakov *et al.*, 2021).

Finally, Couzinet and collaborators showed that IRF-5 promotes apoptosis in the context of DNA damage and tumor suppression. They presented a specific function of IRF-5 that is dependent on the cell type and is related to apoptosis triggered by the death receptor Fas. The study demonstrated that DCs and hepatocytes from mice lacking the *Irf-5* gene exhibit resistance to apoptosis upon exposure to a Fas-activating monoclonal antibody. (Couzinet *et al.*, 2008).

1.4.3 The role of IRF-5 in B cell

The expression and function of IRF-5 was identified in B cells at the same time as it was identified in DCs. The first report showed that the activation of IRF-5 by a specific virus leads to the expression of different interferon alpha genes. IRF-5, when activated by NDV, significantly increased the production of IFNβ in B cells (Barnes *et al.*, 2004).

In the subsequent years, IRF-5 was shown to be involved in B cell activation, proliferation, maturation, differentiation into distinct subsets, and antibody production. Indeed, the absence of IRF-5 impaired the normal generation of germinal center B cells and led to age-related splenomegaly, characterized by a disruption of the normal splenic microarchitecture and an accumulation of a specific $B220^{-}$ B cell subset with impaired functionality. The splenic B cells in *Irf5*$^{-/-}$ mice exhibited decreased plasma cell levels, antibody affinity maturation, and class switch recombination. Moreover, IRF-5 directly regulated the expression of *Prdm1*, the gene encoding

for Blimp-1, a master regulator of plasma cell formation. Additionally, *Irf-5*$^{-/-}$ B cells displayed impaired production of IL-6 in response to TLR7 and TLR9 activation (Lien *et al.*, 2010).

In human, De *et al*. identified IRF-5 as an early regulator of B cell activation and demonstrated that IRF-5 expression is induced in B cells upon TLR9/BCR stimulation. IRF-5 induced activation, proliferation, and differentiation of B cells into specialized cells dedicated to secreting antibodies. They also found that targeted knockdown of IRF-5 resulted in a notable retention of immunoglobulin, reduced proliferation, impaired plasmablast differentiation, and diminished IgG secretion. These observed reductions were primarily attributed to impaired B cell activation and clonal expansion (De *et al.*, 2017)

Other studies confirmed the role of IRF-5 in the production of antibodies and the class switch recombination process. For example, Richez et al. found that lack of IRF-5 decreases the activation of B cells and the antibody production and rescues the *FcγRIIB* $^{-/-}$ and *FcγRIIB* $^{-/-}$ Yaa lupus model from disease. IRF-5 expression had the most pronounced impact on the isotypes associated with pathogenic autoantibodies (Richez *et al.*, 2010).

Savitsky *et al.* demonstrated that IRF-5 controls the process of switching antibodies to IgG2a- and IgG2b, which contributes to lupus-like autoimmunity in mice (Savitsky *et al.*, 2010).The findings of Fang *et al.* support the study of Savitsky *et al.* They found that IRF-5 regulates class switch recombination to IgG2a/c by modulating the expression of the transcription factor Ikaros that is essential for allelic exclusion at the Igk locus. Their results demonstrate that IRF-5 regulates IgG2a/c expression by reducing Ikaros expression (Fang *et al.*, 2012).

In addition, IRF-5 can induce the production of proinflammatory cytokines and chemokines and increase memory B cells. Thackray *et al.* showed that *Irf5*-deficient mice exhibited reduced levels of various proinflammatory cytokines and chemokines, a decrease in the number and activity of immune cells, impaired West Nile virus (WNV)-specific antibody responses, and a lower count of antigen-specific memory B cells and long-lived plasma cells (Thackray *et al.*, 2014). In B cells where IRF-5 was artificially introduced, viral infection triggered the expression of various chemokines, specifically CC chemokines like RANTES, MIP-1α and -β, MCP-1, I-309 (known for attracting monocytes and inhibiting apoptosis in thymic cells), and CXC chemokines such as IL-8 (which activates neutrophils) and IP-10 (Barnes *et al.*, 2002b).

In both humans and mice, IRF-5 in B cells has been associated with autoimmune and inflammatory diseases. Guthridge *et al.* showed that the IRF-5 haplotype associated with lupus affects the B-cell receptor pathway (Guthridge *et al.*, 2012).

In mice, the expression of the *Irf-5* gene is sex-dependent. Indeed, estrogen has been demonstrated to enhance the expression of IRF-5 through ERα, affecting the expression and sub-cellular distribution of the IRF-5 protein in splenic B cells in a sex-dependent manner. As a result, more IRF-5 protein was detected in the nucleus of female B cells than males. These observations could explain how IRF-5 contribute to gender differences in autoimmune disease development (Shen *et al.*, 2010).

Barnes and collaborators described for the first time the function of IRF-5 as a tumor suppressor in B cell lymphoma. They showed that IRF-5 is directly regulated by p53, but its impact on cell cycle regulation and promotion of apoptosis occurs independently of p53. IRF-5 inhibits the growth of B cell lymphoma both through G(2)-M cell cycle arrest and modification of genes associated with growth regulation and apoptosis, including p21, BAK, DAP kinase 2 and BAX (Barnes *et al.*, 2003b; Mori *et al.*, 2002).

Additionally, IRF-5 functions as a pro-apoptotic transcription factor and induces inhibition of cell growth of B cells transformed with Epstein-Barr Virus (EBV). EBV transforms primary adult B cells into continuously growing lymphoblastoid cell lines. IRF-4 mediates this EBV transformation of primary B cells by negatively regulating IRF-5 transcription during EBV transformation (Xu *et al.*, 2011).

1.4.4 The role of IRF-5 in T cell

The literature on the function of IRF-5 in T cells is scarce. However, in recent years there has been a growing understanding of the involvement of IRF-5 in in different developmental and functional aspects of T cells, including the differentiation of T helper cells. IRF-5 can operate intrinsically within Th cells or through its ability to affect other cells such as APCs and B cells, which produce different cytokines that can induce pathways in naïve T cells resulting in modifications of critical gene products for Th cell differentiation.

Ishikawa *et al.* published the first article that showed a direct effect of IRF-5 on T cell during virus infection. Human T cell leukemia virus type 1 (HTLV-1), which is the causative agent of adult T cell leukemia (ATL), induces the upregulation of IRF-5 in T cells. This infection leads to the continual transcription of IRF-5 into three different alternatively spliced isoforms (V1, V3, and V4). This splicing does not occur in uninfected T cell lines. IRF-5, when expressed in uninfected T cells, has a role in promoting the expression of cytokines from the tumor necrosis factor family.

However, In HTLV-1-infected T cells, the expression of IRF-5 mRNA is stimulated, particularly by the viral oncoprotein Tax, which activates the V3 promoter of IRF-5. This Tax-regulated isoform is involved in the development of ATL (Ishikawa *et al.*, 2015).

Paun *et al.* described the expression of IRF-5 in T cells for the first time *in vivo*. They showed that TLR7-mediated activation of IRF-5 during chronic *L. donovani* infection (Paun *et al.*, 2011). In addition, IRF-5 plays a key role in the apoptotic signaling pathway triggered by death receptors (Hu *et al.*, 2009). Fabié *et al.* reported an additional cell-intrinsic role for IRF-5 in $CD4^+$ T cells. This study provided evidence that IRF-5 promotes cell death in IFN-γ^+ $CD4^+$ T cells during chronic *L. donovani* infection. Namely, IRF-5 activation is induced in IFN-γ^+ $CD4^+$ T cells by apoptotic material through TLR7 signalling, leading to the upregulation of factors involved in TRAIL-mediated apoptosis, such as death receptor 5 (DR5) and caspase 8 (Fabié *et al.*, 2018).

IRF-5 was also shown to modulate T cell responses through its interactions with the TCR signaling pathway. Indeed, IRF-5 is essential for the efficient assembly and activation of signaling complexes triggered by the T cell receptor and for subsequent early downstream signaling events. IRF-5 can influence TCR signaling by modulating the expression of key molecules involved in T cell activation. For example, the formation of the SLP76, VAV1, and PLCγ1 complex upon T cell activation is diminished in $CD4^+$ T cells lacking IRF-5. the activation of ZAP70, PLCγ1, and Lck as proximal TCR-initiated signaling pathways and the distal ERK, p38, and JNK signaling pathway, required for T cell activation and differentiation, are reduced in *IRF-5*$^{-/-}$CD4+ T cells (Yan *et al.*, 2020). The same study also revealed that IRF5-deficient $CD4^+$ T cells exhibited a reduction in Th1 and Th17 cytokines (IFN-γ and IL-17) production in contrast to their IRF5-competent counterparts. In contrast, the prevalence of *IRF5*-deficient $CD4^+$ T cells expressing Th2 cytokines (IL-4 and IL-13) was elevated compared to *IRF5*-competent $CD4^+$ T cells *in vivo.* As well, they demonstrated that IRF-5 in $CD4^+$ T cells interacts with promoters that control the expression of cytokines associated with Th1 and Th17 immune responses, promoting T cell-specific cytokine production *in vitro* (Yan et al., 2020).

IRF-5 is also involved in lymphocyte trafficking. Indeed, virus-induced chemokines (RANTES, MCP-1, MIP-1α, MIP-1β, IL-8, I-309, and IP-10) in IRF-5-overexpressing cells have lymphocyte chemotactic activity and IRF-5 expressed on T cells directly affects their migration to lymph nodes (Yan *et al.*, 2020).

Finally, IRF-5 in T cells has also been associated with autoimmune diseases, suggesting a potential role in T cell-mediated pathogenesis. For example, Starskaia *et al.* report 79 differentially methylated regions in $CD4^+$ T cells and 56 in $CD8^+$ T cells in individuals with specific

autoantibodies for type 1 diabetes. Notably, they observed DNA methylation changes associated with the disease at a very early stage, which included differential methylation in the promoter region of *IRF5* in $CD4^+$ T cells (Starskaia *et al.*, 2022).

PART V: HUMAN IMMUNODEFICIENCY VIRUS (HIV)

Awareness of HIV started gaining worldwide attention in the 1980s, primarily due to the identification of cases involving advanced and unexplained immunodeficiency. In the early stages of the epidemic, a notable occurrence was the increased incidence of opportunistic infections and rare cancers in homosexual men in urban centers. This marked the initial recognition of what later became known as AIDS. Subsequently, the identification of HIV as the causative agent for AIDS occurred in 1983 by two independent research groups. The virus was initially named Lymphadenopathy-Associated Virus (LAV) and later called Human T-Cell Lymphotropic Virus III (HTLV-III) (Barré-Sinoussi *et al.*, 1983; Gallo *et al.*, 1983).

HIV infection is characterized by a persistent inflammatory state, which accelerates the progression to AIDS, especially in individuals who are not on combination ART. The human body cannot fully eliminate HIV, even with treatment. Consequently, it remains a lifelong infection, and to date there is no definitive cure for the disease (Mody *et al.*, 2024).

The global prevalence of HIV infection presents a major public health challenge worldwide. According to latest global and regional statistics on the status of the AIDS epidemic from The Joint United Nations Programme on HIV and AIDS (UNAIDS) and World Health organization (WHO), in 2022, around 39 million people globally were living with HIV, 1.3 million people became newly infected with HIV, 85.6 million people have become infected with HIV and 40.4 million people have died from AIDS-related illnesses since the start of the epidemic (UNAIDS, 2023).

1.1 HIV BIOLOGY

HIV belongs to the family *Retroviridae*, subfamily *Orthoretrovirinae* and the genus *Lentivirus*. Non-human primate's immunodeficiency viruses, known as simian immunodeficiency virus (SIV), are also categorized within the *Lentivirus* genus. The emergence of HIV is believed to have originated from non-human primates and then sporadically transmitted to humans; a process known as zoonosis (Sharp *et al.*, 2011).

HIV is classified into two main types, HIV-1 and HIV-2, based on genetic characteristics and differences in viral antigens. Each of these types is further divided into various subtypes and circulating recombinant forms and unique recombinant forms (Faria *et al.*, 2014).

HIV has a relatively simple genome structure, which comprises a pair of identical single-stranded RNA molecules encapsulated within the viral core. The HIV genome with approximately 9.7 kb, encodes three structural proteins, two envelope proteins, three enzymes, and six accessory proteins that play crucial roles in the viral life cycle (Figure 1.5). The group-specific antigen (gag) gene is responsible for encoding 3 structural proteins. The integral outer core membrane protein Matrix (MA, p17) contributes to the formation of the inner membrane layer. Capsid protein (CA, p24) forms the viral core, enclosing the genetic material. Nucleoprotein (NC, p7) is involved in the formation of the Nucleocapsid and binds to the viral RNA to form RNA complex, assisting in packaging into the virion. Budding protein (p6) is a small nucleic acid-stabilizing protein involved in virus particle release (van Heuvel *et al.*, 2022).

Adjacent to the gag gene is the polymerase (pol) gene, which codes for the HIV enzymes. The protease enzyme (PR, p11) is in charge of proteolytic cleavage of precursors and release of structural proteins and viral enzymes. Reverse transcriptase (RT, p51) and RNase H (p15) or RT plus RNase H (together p66) transcribe HIV RNA into proviral DNA. Integrase (IN, p32) integrates proviral DNA into the host genome (Rhee *et al.*, 2016).

The pol gene is followed by the envelope gen (env) reading frame, which encodes for structural proteins, giving rise to two envelope glycoproteins, surface glycoprotein (SU, gp120), which binds to the CD4 receptor on the host cell surface to ensure its adhesion during virus entry, and transmembrane glycoprotein (TM, gp41), which facilitates the anchoring of gp120 and viral and cell membrane fusion. Then there are the HIV genes that encode several accessory or regulatory proteins, including viral infectivity factor (vif), viral protein R (vpr), viral protein U (vpu), trans-activator of transcription (Tat), regulator of virion (Rev) and negative factor (nef). Additionally, HIV produces a Tat-Env-Rev fusion protein (tat/rev protein (tev), p26), which regulates the activity of Tat and Rev in nucleus (Vicenzi *et al.*, 2013).

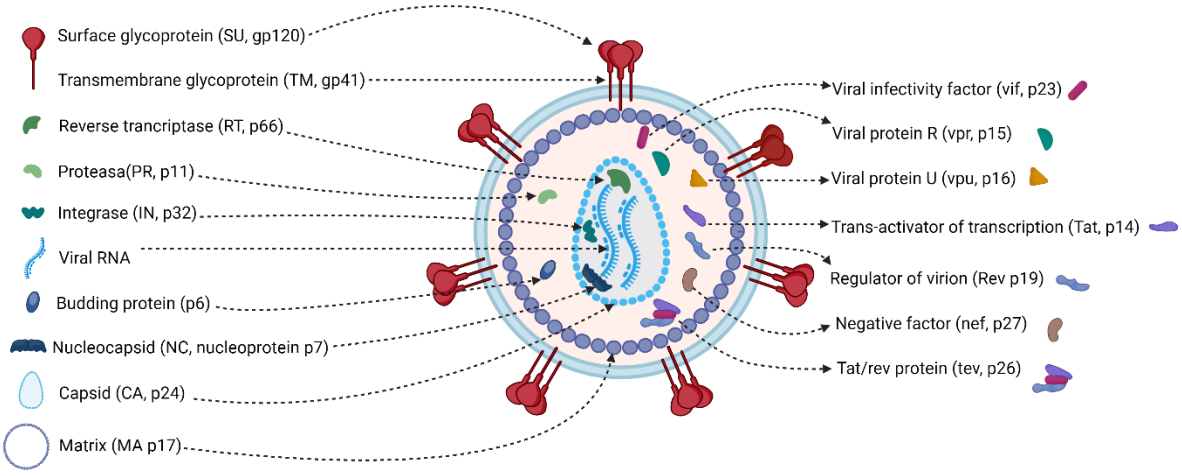

Figure 1.5 HIV virion particles and proteins

Structure of a HIV virion particles. The fully formed enveloped virion exhibits a spherical configuration, encased within a lipid bilayer membrane derived from the host cell. This membrane comprises 7–35 envelope glycoprotein trimers. Inside this membrane, the inner layer anchors the MA proteins derived from Gag and also contains PR and accessory proteins. The capsid, situated at the virion's core, houses two copies of gRNA, RT, and IN. The integrity of the gRNA is maintained by the NC proteins. Created with BioRender.com.

1.2 HIV life cycle

Exposure to HIV occurs primarily via the mucosal route, and the establishment of infection depends on the expression of CD4 and the chemokine receptors CC-chemokine receptor 5 (CCR5) or the CXC-chemokine receptor 4 (CXCR4) on the target cell (Figure 1.6). Initial infection involves complex interactions between HIV Env proteins and the CD4 receptor. The HIV surface glycoprotein gp120 binds to CD4 on the host cell, causing conformational changes in CD4, gp120, and subsequently gp41. This change exposes a site on gp120 for binding to the co-receptor CCR5 or CXCR4, leading to fusion of the viral envelope with the cell membrane and formation of the fusion pore. *In vivo*, CCR5 and CXCR4 are the main receptors used by HIV, with CCR5 being the primary co-receptor for naturally transmitted virus (Xiao *et al.*, 2021).

Upon entry into the cytoplasm of the host cell, the HIV-1 capsid shields viral HIV RNA genome and core proteins, evading detection by intracellular sensors or restriction factors. Within the cytoplasm, HIV RT is activated to transcribe viral RNA genome into DNA. Following RNA

degradation, the resultant double-stranded HIV DNA integrates into the human host cell genome. This integration event concludes the HIV infection of the cell and solidifies the establishment of a persistent infection. The proviral genome has the capacity to replicate in tandem with and as an integral part of the host cell genome during cell division (Pan *et al.*, 2013).

Finally, the complete set of HIV proteins is transcribed and translated, and subsequently transported to the plasma membrane. At the membrane, the virus assembles and exits the cell, completing its maturation process and facilitating the spread of infection (Monje-Galvan *et al.*, 2020).

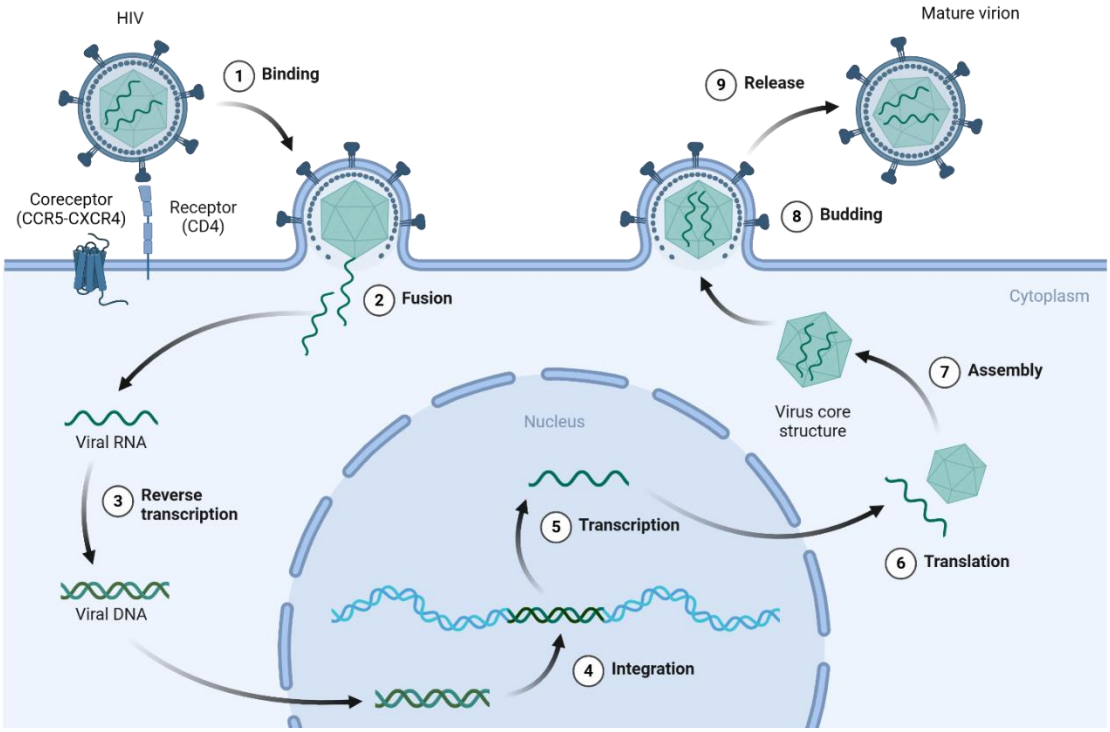

Figure 1.6 HIV replication cycle

HIV gains entry into its target cells by first interacting with the CD4 receptor and either CCR5 or CXCR4 via the envelope (Env) glycoprotein (step 1). HIV fuses with the surface of the host cell and the protective shell of the capsid is ruptured, allowing the genetic material and proteins of the virus to enter the cell (step 2). Following fusion and uncoating, the viral RNA is reverse transcribed into DNA (step 3). The resulting pre-integration complex is transported into the nucleus, where the viral DNA integrates into the host genome (step 4). Host enzymes facilitate the transcription of HIV DNA into viral mRNAs (step 5), which are then exported to the cytoplasm for translation (step 6). This translation process produces viral proteins, leading to the assembly of immature (non-infectious) virions (step 7). At the culmination of the HIV life cycle, known as budding, immature HIV particles emerge from the host cell. These immature viruses lack the ability to infect other cells (step 8). Upon leaving the host cell, the newly formed HIV releases the protease enzyme,

which cleaves the long protein chains of the immature virus, transforming it into a mature, infectious form (step 9). Created with BioRender.com.

1.3 HIV infection progression

HIV infection progresses in stages, with the initial stage being acute HIV infection, followed by the chronic stage. Without proper medical care and treatment, HIV can eventually lead to AIDS, which is the most severe stage of HIV infection (Figure 1.7). Acute HIV infection (also known as primary HIV–infected (PHI) phase) typically presents with flu-like symptoms and is characterized by a high-titer viremia. Then the immune response primarily driven by CTLs and, to some extent, anti-HIV antibodies, swiftly controls this initial viremia (Clarke *et al.*, 2024).

The subsequent phase of HIV infection is characterized as chronic HIV infection. In this phase, the virus persists in the body, viral RNA remains detectable in plasma, the virus replicates at minimal levels and there is active viral turnover, with significant virus production and shedding each day. Individuals with chronic HIV infection might not experience any HIV-related symptoms. However, a failure of immune control causes a significant decrease in the number of $CD4^+$ T lymphocytes and a rapid increase in viral load. It is after this loss of immune control that the $CD4^+$ T cell count drops below 300/µL, viral load increases to over 40 copies/mL and the clinical symptoms of AIDS manifest. AIDS is characterized by severe immune system suppression, leaving the body vulnerable to opportunistic infections and certain types of cancers. Without ART chronic HIV infection typically progresses to AIDS within 10 years or more, although the rate of progression can vary among individuals (Dessie *et al.*, 2020).

Although modern antiretroviral treatments are very effective in controlling HIV, they do not clear the infection, requiring lifelong adherence to maintain viral suppression and immune health. Achieving a true cure is challenging due to the genetic diversity of the virus and rapid mutation, which help it evade immune responses and adapt to treatments. Additionally, HIV can evade the immune system by downregulating viral antigens on infected cells, making it difficult for the immune system to detect and eliminate these cells. (Blassel *et al.*, 2021).

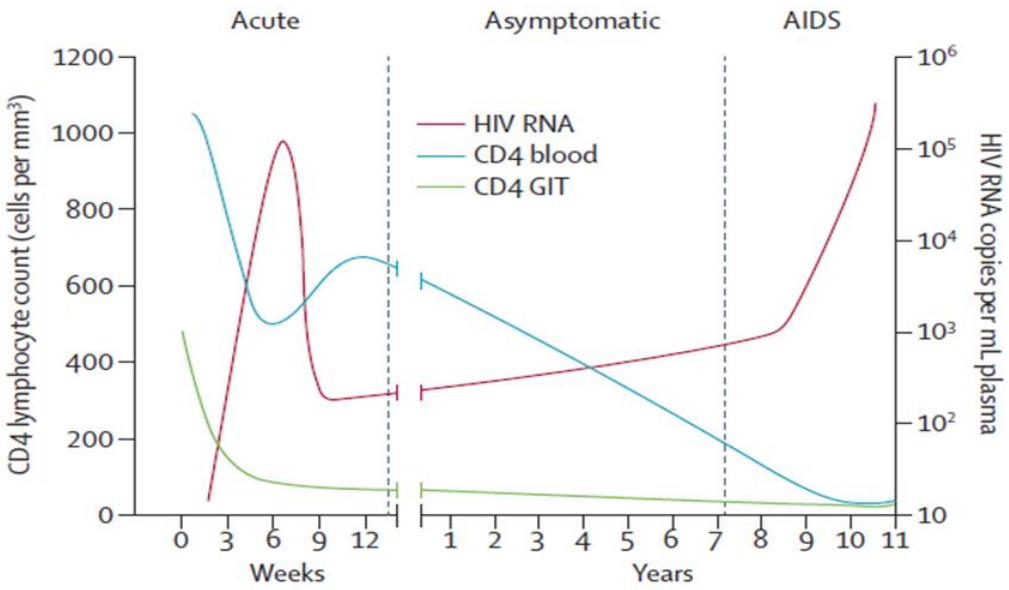

Figure 1.7 Clinical course of HIV Infection

There are three stages of HIV infection: Acute HIV infection, Chronic HIV Infection or asymptomatic, AIDS. Acute HIV infection marks the initial phase of HIV progression. HIV proliferates swiftly during this period, disseminating across the body and targeting $CD4^+$ T cells. With heightened viral activity, blood HIV levels surge, amplifying transmission risk. In chronic HIV infection, also known as asymptomatic HIV infection or clinical latency, the virus persists in the body, albeit at minimal levels. Individuals may remain asymptomatic during this phase. Without ART, chronic HIV infection typically progresses to AIDS over a span of 10 years or more, although the timeline may vary among individuals (Maartens *et al.*, 2014).

Additionally, HIV has the ability to establish latent infections in long-lived cells, such as memory T cells. These latently infected cells, mostly transcriptionally inactive, harbor replication-competent virus, resulting in a resurgence of infection when antiretroviral therapy is discontinued (Margolis, 2022).

Most people living with HIV (PLWH) who receive effective ART experience a restoration of $CD4^+$ T cell counts. This immune recovery is a key indicator of successful HIV treatment. However, some PLWH who, despite receiving ART, exhibit suboptimal immune recovery with $CD4^+$ T-cell counts not rising to expected levels, can experience persistent immune dysfunction despite having suppressed viral loads. This group is called immunological non-responders (INRs) or discordant individuals (Rb-Silva *et al.*, 2019).

The reasons for immunological non-response can be complex and can involve diverse factors. Ongoing immune activation and inflammation, even in the presence of viral suppression, that could impede $CD4^+$ T cell recovery. Older age at the time of HIV diagnosis and a delayed initiation of antiretroviral treatment, especially in advanced stages of HIV infection, can affect the extent of immune recovery. In addition, concurrent infections, particularly chronic viral infections, can contribute to immune dysfunction and influence the response to ART. Viral reservoirs can still exist despite viral suppression, affecting immune restoration (Bono *et al.*, 2022).

Contrary to INRs, there are individuals who have endured HIV infection for an extended period without manifesting symptoms of the disease and without undergoing ART; these individuals are known as long-term non-progressors (LTNPs) or long-term survivors (LTS). LTNPs are asymptomatic for more than 10 years without antiviral therapy, maintain normal $CD4^+$ T cell counts, demonstrate a low viral load (assessed by plasma viremia and infected PBMCs), and exhibit low immune activation and normal T regulatory function (De La Torre-Tarazona *et al.*, 2022). Within LTNPs, a subgroup has been recognized and labeled as elite controllers (EC). These individuals can naturally control the virus without ART, spanning a period of at least 2 years and in some cases exceeding 10 years, exhibit no detectable virus in the plasma despite remaining infected. These individuals exemplify an optimal level of HIV control among long-term survivors and likely share similar immune resistance mechanisms with LTNPs, albeit with more pronounced and effective responses (Capa *et al.*, 2022).

The exact mechanisms underlying the maintenance of effective immunity and tissue integrity despite the presence of inflammation in HIV elite controllers are not fully understood, but appear to involve a combination of genetic, immunological, and virological factors. For instance, a primary determinant is mutations affecting the expression of the CCR5 gene, such as the 32-base pair deletion (CCR5-delta32 genotype) (Claireaux *et al.*, 2022). Additional mutations within genes such as CCR2, CX3CR1, CXCL12 , CCL3L1 and CCL5 (RANTES) have been recognized and linked to either host resistance or susceptibility to HIV-1 infection and the progression of the disease (Reiche *et al.*, 2007). One of the key host factors linked to effective viral control is the human leukocyte antigen (HLA) type. Consequently, individuals with the capacity to generate highly functional HIV-specific T cell responses, characterized by robust proliferative capacity and efficient targeting of favorable epitopes, exhibit elite control (Acevedo-Sáenz *et al.*, 2015).

While various viral and host factors contribute to elite control, immunological factor such as elevated functionality of HIV-specific CD8 T-cells, maintenance of memory and peripheral T-

follicular helper CD4 T-cells, sustained HIV-1-specific B-cell responses, diminished levels of NK cell activation, and the preservation of plasmacytoid dendritic cells (pDC) characterize the immunological profile associated with optimal HIV control. Additionally, reduced disruption of intestinal integrity and dysbiosis of the gut microbiota and metabolism contribute to maintaining local immunity against HIV (Loucif *et al.*, 2018).

PART VI: IMMUNE RESPONSE TO HIV INFECTION

Immune responses and their modulation at different stages of HIV-1 infection are dynamic and involve a complex interplay between various components of the innate and adaptive immune system. Innate immune responses

The innate responses are the initial line of defense, contributing significantly to the early control of virus replication during acute HIV infection. DCs are essential in the control of HIV infection, in that DCs recognize HIV RNA through TLR7 and TLR8 (Wang *et al.*, 2007). This interaction triggers a surge of proinflammatory and antiviral cytokines and chemokines, including IFN-I, TNF, IL-6, CXCL10, CCL4, and CCL5 (Heil *et al.*, 2004). However, DCs can contribute to viral dissemination and $CD4^+$ T cells destruction. Infected DCs can transport the virus to lymphoid tissues and transmit it to $CD4^+$ T cells (Pope *et al.*, 1994).

Monocytes and macrophages can also recognize HIV components through various TLRs present on their surfaces or within their cellular compartments (Kedzierska *et al.*, 2002). This recognition triggers their activation, bringing them into a proinflammatory state and promoting functions important for HIV control, such as antigen presentation, T and B cell coactivation, cytokine production, phagocytosis, and chemotaxis. However, in the later stages of HIV infection, the functionality of these cells is compromised, and they also act as viral reservoirs, contributing to the chronic nature of the infection (Wacleche *et al.*, 2018)

Similar to mononuclear phagocytes, NK cells can directly recognize and eliminate HIV-infected cells through cytotoxic mechanisms, inducing apoptosis or recognizing antibody-coated HIV-infected cells through their Fc receptors, promoting the destruction of these cells through antibody-dependent cell-mediated cytotoxicity (ADCC) (Bernard *et al.*, 2022). Numerous studies indicate that HIV-1 significantly alters the balance of NK cell populations and impairs their ability to function effectively against the virus. Elevated chronic HIV-1 viremia often leads to a

dysfunctional redistribution of NK cell subsets, characterized by an increase in anergic CD56/CD16 NK cells (Mavilio *et al.*, 2005)

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1.1 IFN-I role in HIV immunity

IFNs occupy a central place in the antiviral defense mechanism. They play a key role in inducing antiviral proteins, regulating signaling events, facilitating the maturation of DCs and activating macrophages, NK cells, B cells and T cells to mount an effective immune response (Stacey *et al.*, 2009). In particular, IFN-I triggers a signaling cascade that prompts adjacent cells to enter an antiviral state. This state involves the upregulation of various antiviral proteins and the inhibition of viral replication. For example, some important antiviral proteins that interferes with HIV infection are listed in Table 1.5 (Schoggins *et al.*, 2011).

Table 1.6 Antiviral proteins that interferes with HIV infection

ISG gen	Funtion	Mechanism
ISG20	Inhibition of viral replication	Degradation of Viral RNA by 3'-5' exonuclease activity
ISG15	Inhibition of release of newly formed HIV-1 virions	Inhibition of assembly and budding of new HIV virions by Interfering with HIV Gag protein
OAS1	Inhibition of viral replication	Activation of the latent ribonuclease, RNase L, which in turn degrades viral RNA, inhibiting viral replication
ADAR	Inhibition of viral replication	Modification of HIV-1 envelope glycoproteins RNA sequence by RNA editing enzyme activity in macrophages but not in T cells
APOBEC3G/3F	Inhibition of viral replication	Production of mutations in the minus strand DNA during reverse transcription to prevent proviral integration in T cells.

Tetherin, or CD317 or BST2	Inhibition of release of newly formed HIV-1 virions	Prevention of virion release by forming a physical bridge between budding virions and the host cell membrane
IFITM1/2/3	Inhibition of HIV-1 entry	IFITMs to inhibit cell-to-cell infection by antagonizing the HIV-1 envelope glycoprotein (Env)
MOV10	Inhibition of viral replication	Reduction of virus production by inhibition of proteolytic processing of HIV-1 Gag, and reverse transcription
MX2 (MxB)	Inhibition of viral replication	Restriction of HIV-1 nuclear import by binding to the core and inhibits the uncoating process. Inhibition of HIV-1 DNA integration by destabilizing HIV-1 nuclear DNA.
TRIM5	Inhibition of viral replication	Restriction of HIV-1 infection by binding the viral capsid, blocking HIV-1 disclosure, and inducing viral RNA degradation
SUN2 (UNC84B)	Inhibition of viral replication	Modification of nuclear envelope shape and viral infection HIV is blocked after reverse transcription at or before nuclear import
GBP1,5	Inhibition of release of newly formed HIV-1 virions	Interference of viral envelope glycoprotein (Env) processing and incorporation into the virion

Despite the efficiency of IFN-I and antiviral restriction factors in protecting cells from HIV infection, they cannot completely control the infection because the virus has developed various methods to evade the host's defense mechanisms. HIV-encoded proteins interfere with cellular pathways involved in IFN production or signaling. For instance, Vpu can inhibit the expression of IRF-3, and Vpx can inhibit IRF-5 function (Cheng *et al.*, 2014; Doehle *et al.*, 2012). Additionally, Vpu and Nef can interfere with JAK-STAT signaling pathway, critical for IFN responses, thereby dampening the cellular response to IFNs (Nguyen *et al.*, 2018).

Although IFN-I is a cytokine that plays a pivotal role in antiviral responses it can also contribute to immune system activation and tissue damage when chronically elevated (Harris *et al.*, 2010). Sustained IFN-I signaling is increasingly recognized as a central mechanism driving persistent inflammation that is associated with impaired disease progression in chronic HIV infection (Klatt *et al.*, 2013).

1.2 Adaptive immune responses

A strong and effective adaptive immune response helps reduce viral load, which correlates with improved clinical outcomes and slows disease progression, reducing susceptibility to opportunistic infections and complications associated with advanced HIV disease (Brenna *et al.*, 2022; Koup *et al.*, 1994). However, in most individuals the adaptive immune response in HIV infection is insufficient or delayed, meaning that by the time it develops, it is often too late to completely eradicate the infection. Only LTNP or EC individuals develop adaptive humoral and cellular responses, that control the infection (Fonseca *et al.*, 2011).

1.2.1 T cells during HIV infection

The adaptive immune system relies heavily on T cell responses to eliminate infected cells. When activated, these T cells can effectively destroy target cells. Nevertheless, during the course of infection, the balance of various T cell populations is altered. This disruption not only affects the short-lived cells that are naturally part of the immune response, but also puts pressure on the maintenance of resting naive and memory T cell pools (Gordon *et al.*, 2010).

In HIV chronic infection, constant exposure to high levels of antigens causes T cells to enter a dysfunctional state known as exhaustion, which impairs their ability to function effectively (Fenwick *et al.*, 2019). The hyperactivated or exhausted phenotype is often marked by increased expression of certain markers, like programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), lymphocyte-activation gene 3 (LAG-3), T-cell immunoglobulin and mucin domain-containing protein 3 (TIM-3), killer cell lectin-like receptor subfamily G member 1 (KLRG1), T cell immunoreceptor with Ig and ITIM domains (TIGIT), among others (Wang *et al.*, 2020). The presence of exhausted T cells is notably elevated within the gut-associated lymphoid tissues (GALT), the primary location for viral replication, as compared with levels observed in the peripheral blood. In addition, these cells have a reduced proliferative capacity, altered cytokine production, and impaired effector functions (Rueda *et al.*, 2012).

Despite ART, a persistent chronic immune activation and inflammation linger throughout the disease. This ongoing immune activation, deemed an accelerant for non-AIDS-related

complications and a contributor to $CD4^+$ T cell depletion, remains a challenge. The intricate mechanisms behind this excessive inflammation in HIV remain yet to be fully elucidated (Lv *et al.*, 2021b).

1.2.1.1 $CD8^+$ T cells during HIV infection

$CD8^+$ T-cells are pivotal in the cellular immune response, crucially contributing to the control and containment of HIV infection during acute infection. $CD8^+$ CTLs detect infected cells by an MHC-I-dependent mechanism, enabling them to eliminate infected cells by releasing cytotoxic molecules such as perforin and granzymes, or by interacting with cell surface death receptors. HIV-1-specific CTLs responses contribute to the daily killing of up to 30% of virus-infected cells (Goonetilleke *et al.*, 2009). Additionally, $CD8^+$ T cells release β-chemokines and antiviral factor that hinder viral processes, impede viral binding, and interfere with viral transcription (Morvan *et al.*, 2021).

It is important to note that the frequency and proportion of $CD8^+$ T cells that are polyfunctional, producing and secreting cytokines such as IFN-γ, TNF, IL-2, in addition to killing target cells or having a non-cytotoxic function, are inversely proportional to viral load in the early stages of infection (Betts *et al.*, 2006).

Nevertheless, in most cases, the $CD8^+$ T cell response falls short in maintaining long-term control of viremia. Indeed, persistent viremia contributes to the development of generalized $CD8^+$ T cell dysfunction, characterized by a decline in proliferative and cytolytic capabilities (Eller *et al.*, 2016). HIV induces altered $CD8^+$ T cell function through different mechanisms. HIV rapid mutation rate enables evasion of $CD8^+$ T-cell recognition, alongside down-regulation of MHC-I expression on infected cells. Additionally, HIV disrupts $CD8^+$ T cell signaling by altering cytokine production and cellular receptor engagement. This disrupted TCR stimulation induces an anergic state in these cells. Moreover, HIV impacts $CD4^+$ T cells and antigen-presenting cells, necessary for proper $CD8^+$ T-cell maturation, ultimately reducing the number of effective $CD8^+$ T cells available to combat the virus (Gulzar *et al.*, 2004).

1.2.1.2 $CD4^+$ T cells during HIV infection

Virus-specific helper $CD4^+$ T cell responses contribute to the maintenance and efficacy of robust immunity to HIV infection. In individuals controlling viral levels without antiviral therapies, persistent and robust HIV-specific $CD4^+$ T cell responses are observed (Rosenberg *et al.*, 1997).

The production of cytokines by different types of Th cells plays a crucial role in the pathogenesis of HIV infection. In both the acute and chronic stages of the infection, serum concentrations of specific cytokines are found, including IFNs, IL-10, IL-4, TNF, IL-2, IL -6, IL-13 and IL-22 (Gorenec *et al.*, 2016). This cytokine storms profoundly affect T cell responses and lead to increased immune activation, potentially exacerbating $CD4^+$ T cell depletion, influencing their function and proliferation, and contributing to the dysregulation of the immune system. It also affects the balance of T cell subsets and their cytokine production, leading to an unfavorable environment for controlling HIV replication (Muema *et al.*, 2020).

PLWH displaying lower immune activation show increased frequencies of $CD4^+$ T cells producing IFN-γ or IL-17 and higher ratios of effector-to-regulatory cells (Chevalier *et al.*, 2016). Particularly, HIV-1- p24-specific Th1 responses, marked by the production of IFN-γ, IL-2 and antiviral β-chemokines, have an inverse correlation with viral load. Moreover, robust Th1 proliferative reactions to HIV-1 are evident when acutely infected individuals are treated with antiviral therapy (Ndongala *et al.*, 2009). IFN-γ is known to promote antiviral activity and Th1 cells assist in the development of immunological memory and help activate cytotoxic $CD8^+$ T cells and APCs, to target and eliminate HIV-infected cells (Kubo *et al.*, 2016). However, long-term overproduction of IFN-γ in HIV infection leads to inflammation and tissue damage (Cordeiro *et al.*, 2022). In PLWH, the frequency of HIV-specific Th1 cells and IFN-γ production significantly declines after ART, but early treatment initiation is linked to better preservation of these cells (Chevalier *et al.*, 2016).

It is hypothesized that the loss of Th1 response and the continued progression of HIV infection to AIDS is due in part to the fact that HIV causes a shift from a Th1 to a Th2 immune response (Rook *et al.*, 1993). HIV-1 envelope proteins, such as gp160 and gp120, reduce Th1-type responses (IFN-γ /IL-2) and favor dominant Th2-type responses characterized by extensive IL-4 production (Hu *et al.*, 1994). The establishment of a type 2 dominant response favors virus replication and virus spread. In certain HIV-positive individuals with diminished $CD4^+$ T cell counts, there is an observed prevalence of T cells generating type 2 cytokines, evident in both peripheral blood and skin (Romagnani *et al.*, 1994).

Tfh cells play a fundamental role in the development of humoral responses against HIV. Within the follicles of secondary lymphoid organs, these cells assist cognate B cells by facilitating interactions through ligand-receptors and soluble factors essential for promoting germinal center (GC) expansion, guiding the maturation of B cells into antibody-secreting cells (ASCs), and promoting the creation of high-affinity antibodies, including broadly neutralizing antibodies (bNAbs) (Moysi *et al.*, 2018). Nevertheless, in individuals with chronic HIV infection, compromised GC-Tfh cell function has been observed, leading to inadequate support for GC-B cells (Cubas *et al.*, 2013).

Tregs possess a dual role in HIV infection. While they can suppress anti-HIV specific responses, potentially hindering the immune system fight against the virus, they also have a beneficial function by mitigating the damaging effects of excessive immune activation preventing immunopathology (Jenabian *et al.*, 2012).

Th17 and Th22 exhibit overlapping functionalities in mucosal compartments, especially in GALT, where HIV primarily replicates, disrupting the mucosa and causing a decrease in $CD4^+$ T cells within the gut, which alters the microbial balance and allows microbial products to enter the bloodstream, leading to dysbiosis. The frequencies of Th17 and Th22 cells in the intestine are positively correlated with intestinal $CD4^+$ T cell ratios and inversely correlated with intestinal mucosal damage, as well as with plasma viral loads. Their decrease in the intestinal region is correlated with local mucosal immune dysfunction during persistent HIV infection (Ancuta *et al.*, 2010; Xu *et al.*, 2014).

In recent research a distinct subset of cytolytic $CD4^+$ T cells has emerged, demonstrating substantial killing capacity similar to that of HIV-specific $CD8^+$ T cells. Their collaborative efforts play an important role in targeting and eliminating virus-infected cells (Johnson *et al.*, 2015).

Finally, one of the most important subtypes of T cells in adaptive immunity are the memory T lymphocytes. The memory $CD4^+$ T cell subset is preferentially infected by the virus, which causes selective functional defects and produces a great loss of this subset of $CD4^+$ T cells. After the partial control of the infection, the virus replicates rapidly, spreads widely, and there is significant depletion of more than fifty percent of memory $CD4^+$ T cells in various tissue compartments, indicating substantial and irreparable damage to the immune system (Gartner *et al.*, 2020; Schnittman *et al.*, 1990). $CD4^+$ T cell depletion, especially in mucosal areas, occurs early in HIV infections and is not fully reversed by ART, especially if initiated during compromised T cell function. The virus targets and eliminates activated CCR5-expressing effector and memory $CD4^+$ T cells in the intestinal lamina propria. This depletion is substantial regardless of infection

progression and predicts clinical outcomes. While early ART does not prevent initial mucosal $CD4^+$ T cells depletion, it significantly aids in their recovery. However, complete restoration of these cells is slow, even with controlled virus and reduced inflammation (Le Hingrat *et al.*, 2021). Indeed, after the subsequent increase in $CD4^+$ T cells counts with ART, the restoration of antigen specific $CD4^+$ T cells cell memory from vaccinations or prior infections did not occur (Thomas *et al.*, 2020). Moreover, some individuals have an irreversible loss of $CD4^+$ T cells skin tissue Resident Memory T (Trm) cells and face an increased risk of skin and mucosal cancers (Saluzzo *et al.*, 2021).

During HIV infection memory cells also show abnormal maturation and defective functions. In the majority of individuals, HIV-1-specific $CD4^+$ T cells exhibit a bias toward an initial central memory phenotype. Furthermore, post-ART, these $CD4^+$ T cells specific to HIV-1 demonstrate a prevalence of $CD27^+CD28^-$ expression, which represents an uncommon phenotype and indicates an early intermediate stage of differentiation (Yue *et al.*, 2004).

Individuals who have the ability to maintain a good number of functional memory T lymphocytes, have a better prognosis in the development of the disease. For instance, EC individuals have a higher percentages of memory cells, especially with less differentiated phenotypes like central memory T (Tcm) cells, and lower percentages of cells with differentiated phenotypes like and effector memory (Tem) and terminally differentiated effector memory (TEMRA) cells (Meraviglia *et al.*, 2019).

1.2.1.3 $CD4^+$ T cell and latent infection

While $CD4^+$ T cells, contribute to controlling HIV infection, they also play a role in perpetuating viral persistence. The different $CD4^+$ T cell memory subsets also play unique roles in the establishment of latent infection. Each memory subsets exhibiting varying susceptibilities to HIV-1 infection. This susceptibility is influenced by distinct cellular activation states, tissue localization, and coreceptor expression (Morcilla *et al.*, 2021). HIV initially aims at a select group of rapidly dividing memory $CD4^+$ T cells that showcase prominent surface expression of CCR5 (Gantner *et al.*, 2023). Before treatment, during the acute infection, infected cells are more likely to exhibit characteristics of transitional memory (Ttm) cells and are abundant in subsets like Th17, peripheral Tfh, and regulatory T cells. They notably show increased markers of activation (CD25, HLA-DR, Ki67), immune checkpoint molecules (PD-1, LAG-3, TIGIT, Tim-3), as well as integrins α4β7 and α4β1 (Pardons *et al.*, 2019). In individuals with chronic untreated HIV-1, long-lived

resting memory $CD4^+$ T lymphocytes, such as Tcm, are the primary targets of latent infection (Brenchley *et al.*, 2004).

In PLWH on ART reservoir exhibit characteristics of Tem, Ttm and Tcm. These cells also expressed immune checkpoint molecules like PD-1, TIGIT, along with the integrin α4β1 (Pardons *et al.*, 2019). In addition, $CD4^+$ T cells expressing the chemokine receptor CCR6 have been identified as a reservoir for lingering HIV in gut tissues (Anderson *et al.*, 2020). Tfh, Th1, Th17 and Th22 were also reported to be an HIV reservoir even in the context of ART (Banga *et al.*, 2016; Sun *et al.*, 2015; Wacleche *et al.*, 2016).

T cell survival and homeostatic proliferation are crucial mechanisms in maintaining immunological memory but they contribute significantly to the persistence of latently infected memory $CD4^+$ T cells in PLWH (Cattin *et al.*, 2022; Chomont *et al.*, 2011). Even with long-term effective ART, the virus persists in cells, particularly in memory CD4 T cells, acting as an enduring reservoir of infectious virus that necessitates continuous lifelong treatment. To reduce the formation of new reservoirs and eliminate latent infected cells, HIV latency reversal agents (LRAs), such as histone deacetylase inhibitors and protein kinase C agonists, that alter epigenetic regulation or cell signaling pathways have been used to activate latent HIV to expose infected cells to the immune system or other antiretroviral drugs and ultimately target and eliminate the hidden virus (Lopes *et al.*, 2021). However, to date, none have managed to reduce the viral reservoir or prolong viral remission after treatment cessation (Deeks *et al.*, 2021).

1.2.2 B cells during HIV infection

Alongside the cellular immune reaction, the body promptly triggers the humoral immune response upon infection. This humoral response encompasses two major classes of antibodies in their action against HIV-1: first, neutralizing antibodies (NAbs) that hinder infection by binding to the envelope of free viruses; and second, non-NAbs that recognize the envelope either during virus entry or on the surface of infected cells (Overbaugh *et al.*, 2012).

Antibody responses against HIV are often ineffective. Indeed, most antibodies that bind Env do not function to neutralize the virus, due to the rapid ability of the virus to evade them (Wei *et al.*, 2003). Nevertheless, about 20–30% of HIV-1 infected individuals, can generate bNAbs. These antibodies have the unique ability to target specific and highly conserved neutralization-sensitive epitopes present on Env trimers (Hraber *et al.*, 2014). These bNAbs can identify either protein-

based epitopes or focus on glycan structures. Unlike most induced neutralizing antibodies, these bNAbs have the remarkable capability to neutralize a wide spectrum of HIV variants, regardless of their specific strains or variations (Binley *et al.*, 2004).

Several bNAbs discovered in untreated HIV-positive individuals target accessible regions such as the CD4 binding site and variable loops of the envelope protein. These discoveries are now being explored for various prevention, treatment, and potential cure approaches (Caskey *et al.*, 2019).

1.3 Mechanisms of cell death in HIV

The detrimental impact of HIV on the immune system derives from its infection and subsequent destruction of Th lymphocytes. Cell death can be induced by the virus or by cytotoxic molecules or it can be receptor-mediated (Figure 1.8). HIV can directly induce cell death of the host cell by different mechanisms. Some HIV strains induce fusion of cell membranes, resulting in the formation of multinucleated syncytia, which reduce the cell lifespan. Moreover, the continuous budding of the virion can compromise cell viability by causing disruption or increased permeability of the plasma membrane. In addition, HIV-infected $CD4^+$T cells can be eliminated from the body by CTLs (Levy, 1993).

Notably, over 95% of the dying cells are not actively infected but rather represent bystander cells. Some studies reveal that the demise of these "bystander" cells is linked to abortive HIV infection (Doitsh *et al.*, 2010). Death of the infected $CD4^+$ T cells also occurs through the LCD. The protein DRAM1, which is regulated by p53 in response to DNA damage, also plays a significant role in promoting Lysosome-dependent cell death in HIV1-infected T cells by connecting Lysosomal membrane permeabilization to membrane permeabilization (Laforge *et al.*, 2013) .

Pyroptosis further contributes to the loss of $CD4^+$ T cells. Immune cells recognize viral components, such as HIV RNA or DNA, which activate inflammasomes and pyroptosis signaling pathways. It sets up a deleterious cycle in which dying $CD4^+$ T cells emit inflammatory signals, which attracts more cells to undergo this process and promotes another type of RCD such as apoptosis (Lao *et al.*, 2022).

Apoptosis plays a very important role in HIV infection. HIV infections disrupt the balance of the entire immune system, possibly contributing to changes in homeostasis, partially through apoptosis. The levels of apoptosis observed in PLWH correlates with the stage of HIV disease

(Cummins *et al.*, 2010a). Initial investigations demonstrated that HIV triggers premature apoptosis in immune cells within lymphoid organs, impacting various subsets such as B cells, $CD4^+$ and $CD8^+$ T cells, as well as dendritic cells (Meyaard *et al.*, 1992). In models leading to AIDS, elevated levels of apoptosis were noted in $CD4^+$ T cells, while enhanced $CD8^+$ T cell apoptosis was observed in both pathogenic and non-pathogenic primate models (Davis *et al.*, 1998). Interestingly, most cells undergoing apoptosis are uninfected during the asymptomatic phase of HIV infection. Apoptosis of uninfected bystander cells is primarily mediated through Fas/FasL interactions. Therefore, the depletion of $CD4^+$ T cells during AIDS is thought to be inadequately explained by the death of cells directly infected with HIV (Dockrell *et al.*, 1999; Finkel *et al.*, 1995).

HIV-1 infection can induce apoptosis through diverse mechanisms, involving complex interactions between the virus and host cells, as well as the activation of inflammatory and immune responses. Some HIV proteins have the ability to directly interact with mitochondria to regulate the process of apoptosis and MPT-driven RCD. For instance, HIV transactivator protein (Tat) causes rapid dissipation of the mitochondrial transmembrane potential and release of cytochrome c in mitochondria, as well as inhibiting substrate oxidation in mitochondria and cytochrome c oxidase (COX) activity (Lecoeur *et al.*, 2012).

Moreover, HIV can induce indirect apoptosis in uninfected T cells mediated by upregulation of FasL on antigen-presenting cells and T cells (Badley *et al.*, 1996). HIV even induces bystander apoptosis through Env glycoprotein and immune activation (Garg *et al.*, 2012). For instance, inappropriate signaling triggered by the binding of the HIV-1 envelope to CD4 can induce aberrant programmed cell death in $CD4^+$ T cells. This is evidenced by the observed *in vitro* cytopathic effect of HIV-1, which is attributed to the induction of programmed cell death mediated by HIV Env protein. This is because the activation of CD4 molecules initiates BAX expression, leading to subsequent dissipation of the mitochondrial membrane potential, release of apoptogenic intermembrane proteins, such as AIF and cytochrome c, caspase activation, and nuclear chromatin condensation (Ferri *et al.*, 2000).

Inactive HIV virions and released HIV proteins in the extracellular environment can exert profound effects on uninfected cells. For example, the HIV protein gp120, soluble and membrane-bound, activate apoptotic pathways, including Fas-dependent (upregulation of Fas/FasL) and Fas-independent (increased Bax, decreased Bcl-2) pathways, through cell receptors such as CD4, CXCR4, and CCR5 (Algeciras-Schimnich *et al.*, 2002; Banda *et al.*, 1992).

The extracellular release of the HIV Nef and Vpu proteins also have been found to induce cell death in various blood cells through a mechanism that is independent of Fas signaling. The

myristylated N terminus of Nef is a significant contributor to its toxicity, as it can insert into the plasma membrane and trigger cell death in uninfected cells. The C terminus of Vpu cause membrane disruption and induce cell death (Azad, 2000). In addition, Vpu induces a caspase-dependent partial cleavage of IRF3, blocking IFN-I responses and inducing apoptosis (Doehle *et al.*, 2012).

The HIV protein Tat also has demonstrated the ability to induce cell death in uninfected cells. HIV Tat has bystander toxicity properties, which involves the accumulation of Tat inside the cell and its release into the extracellular space after infection. Tat interacts with several surface receptors, facilitating cellular uptake by endocytosis. This interaction can result in chromatin remodeling and transcriptional regulation of gene expression in the nucleus, along with down-regulation of receptors, alterations in cytoskeleton organization and induction of apoptosis (Ajasin *et al.*, 2020). Tat leads to apoptosis of Th cells by enhancing the expression of caspase 8 and FasL, via transactivation of the promoters through Egr. This mechanism ultimately leads to increased AICD. Tat can also enhance TRAIL expression on pDCs and monocytes, potentially promoting apoptosis in uninfected T cells (Bartz *et al.*, 1999; Zhang *et al.*, 2001).

The demise of uninfected $CD4^+$ T cells is influenced by the indirect harmful impacts of immune activation. Peripheral T cells from individuals with HIV infection exhibit elevated levels of activation-induced cell death (AICD). Cells undergoing apoptosis show an activated phenotype, marked by features such as HLA-DR positivity, CD38 expression, and carriage of CD45RO and Fas markers. In particular, in HIV-positive individuals, the subset of memory cells expressing CD45RO appear to be particularly susceptible to apoptosis (Gougeon *et al.*, 1996). Actually, apoptosis significantly impacts the loss of memory cells during antigenic stimulation at the same time as CD4 ligation (Howie *et al.*, 1994). AICD in uninfected cells is a natural, multi-step regulatory process that prepares a cell for death, ultimately limiting an activated immune response. Priming can be accomplished through repeated stimulation via CD3/TCR, exclusive stimulation through the CD4 receptor, or activation without co-stimulation. During HIV infection, excessive immune activation can induce apoptosis through the Fas/FasL pathway or through Fas-independent pathways involving CD30 and other members of TNF/NGF receptor family, such as TRAIL receptors (TRAILR1-R4) (Katsikis *et al.*, 1997; Katsikis *et al.*, 1996).

Additionally, HIV proteins can directly regulate other types of RCD. For example, Tat is also implicated in the induction of ferroptosis and ADCD. When primary cells are exposed to the HIV-1 Tat protein, it triggers the initiation of ferroptosis. (Xiao *et al.*, 2022). Recent research has revealed instances of ferroptosis occurring in untreated HIV-1 infected $CD4^+$ T cells and those experiencing HIV-associated neurocognitive disorders. Patients experiencing these conditions were observed to have irregularities or impairments in their mitochondrial function (Sfera *et al.*, 2022).

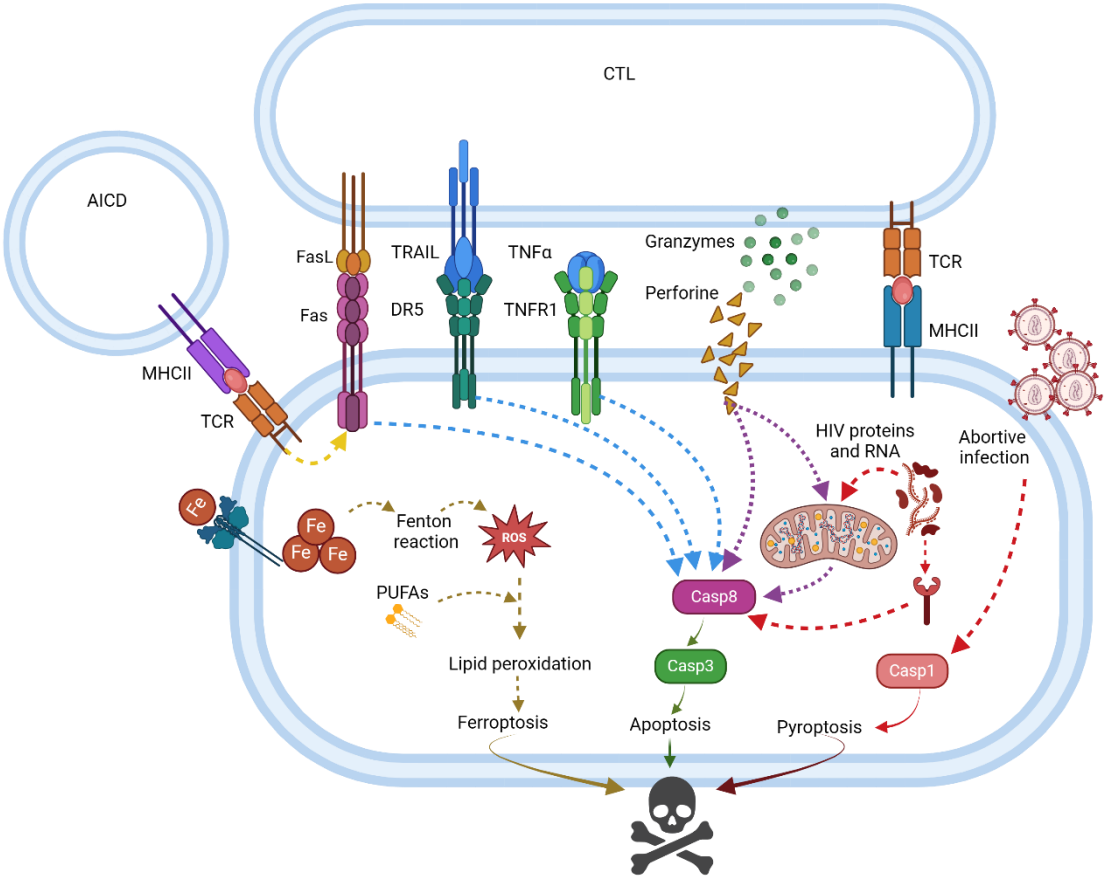

Figure 1.8 Cell death pathways during primary HIV-1 infection

Different types of cell death are the cause of the decrease in of CD4+ T cell during primary HIV-1 infection. Apoptosis, involving extrinsic and intrinsic pathways, is a critical mechanism leading to cell death during all phases of infection. Extrinsic apoptosis entails the engagement of death receptor members within the tumor necrosis factor receptor gene superfamily, such as Fas, TNFR1, and DR5 (TRAILR2). Activation of these receptors by death ligands prompts Caspase 8 activation. Additionally, extrinsic cell death pathways encompass activation-induced cell death (AICD) and the CTLs, which release perforin and granzymes. Intrinsic apoptosis involves Caspase 3 activation induced by HIV proteins and DNA. Additionally, ferroptosis represents another form of CD4+ T cell death instigated by an overabundance of iron-linked lipid peroxides within the cell. The excessive lipid peroxidation leads to the destruction of

cell membranes and ultimately to cell death. Furthermore, abortively infected CD4+ T cells undergo pyroptosis, an intensely inflammatory programmed cell death resulting from Caspase 1 activation. Created with BioRender.com.

1.4 After ART

Early administration of antiretroviral therapy to individuals with HIV has significantly slowed $CD4^+$ T cells loss, disease advancement and mortality rates, particularly, by the reduction of viral replication and related induction of cell death. The decrease in persistent antigenic stimulation and the lack of cellular signals triggered by viral components help in preserving T lymphocytes from apoptosis. This preservation process aids in bolstering immunological revival among individuals undergoing ART treatment (Chavan *et al.*, 1999).

However, T cell susceptibility to cell death is not completely restored by ART. Alterations in apoptosis during antiretroviral therapy substantiate the association between disease progression and apoptosis (Cummins *et al.*, 2010a). In PLWH under ART, the levels of $CD4^+$ T cells displaying early apoptosis is elevated compared with uninfected individuals. Despite increased $CD4^+$ T cell counts, there is no observed decline in early T cell apoptosis in the initial weeks of ART. Interestingly, there is no discernible difference in Fas expression on early apoptotic T cells. This suggests that the immediate expansion of $CD4^+$ T cells during ART is likely not solely due to a diminished rate of early apoptosis among peripheral blood $CD4^+$ T cells (Aries *et al.*, 1998).

After ART, $CD4^+$ T cell death primarily occurs via Fas/FasL-mediated apoptosis, with pyroptosis resulting from abortive infections playing a lesser role. Apoptosis of $CD4^+$ T cells continues to be observed among the memory subset (McCloskey *et al.*, 1998; van Grevenynghe *et al.*, 2008c). Additionally, apoptosis of uninfected CD4 T cells in tonsillar lymphoid tissue also exhibits a memory phenotype and remains predominantly Fas-mediated (Dyrhol-Riise *et al.*, 2001). A significant distinction in the susceptibility to Fas-mediated apoptosis has been observed between PLWH-derived Tcm and Tem under ART compared with EC individuals or HIV^- donors. Specifically, Tcm and Tem cells from PLWH under ART exhibit enhanced susceptibility to Fas-mediated apoptosis. In contrast, memory cells from EC subjects exhibit enhanced resistance to Fas-mediated apoptosis and prolonged persistence after multiple rounds of T cell receptor triggering. The prolonged survival of Tcm and Tem in EC subjects results from the inactivation of the Forkhead box transcription factor O class 3a (Foxo3a) pathway (van Grevenynghe *et al.*, 2008b).

Despite the considerable decrease in various markers of immune activation with ART, residual immune activation persists at significantly higher levels compared to uninfected controls. One of the factors influencing the continuous immune activation is the microbial dysbiosis (French *et al.*, 2009).

The alteration in gut microbiota is extensively documented in PLWH undergoing ART compared with their healthy counterparts. PLWH experience prolonged immune dysregulation and disturbances in microbial balance, contributing to persistent immune activation, inflammation, and cell death (Vadaq *et al.*, 2023; Zhang *et al.*, 2023). The mucosal lymphoid tissue harbors a high concentration of activated memory $CD4^+$ T cells. Within the population of gut memory $CD4^+$ T cells, Th17 cells serves to uphold immune balance and safeguard the integrity of the gut mucosal epithelial barrier, shielding it from microbial infiltration. The severe reduction in gut memory $CD4^+$ T cells and Th17 cells results in the breakdown of the gut mucosal immune system, leading to intestinal dysfunction. This includes compromised mucosal defense against luminal bacteria and deterioration of the integrity of the mucosal epithelial barrier (Bixler *et al.*, 2013). Subsequently, numerous intestinal microbes and their by-products enter the bloodstream, triggering immune activation and an exaggerated inflammatory reaction (Dinh *et al.*, 2015).

This continuous inflammatory cycle further targets $CD4^+$ T cells, making them susceptible to viral infection or apoptosis, thus accelerating their depletion. The loss of these cells significantly heightens vulnerability to opportunistic infections, cancers, and various associated health issues (Brenchley *et al.*, 2006).

Continuous apoptosis in individuals infected with HIV-1 and undergoing ART is linked to a less robust recovery of CD4 T lymphocytes, potentially hampering immune reconstitution (Hansjee *et al.*, 2004). Ongoing accelerated $CD4^+$ T cell apoptosis continues in individuals not responding to immune therapies, even among those achieving full viral suppression on ART (Pitrak *et al.*, 2001). Susceptibility to apoptosis is associated with ongoing immune activation and immunosenescence or attributed to the negative impact of ART on lymphocyte survival via activation of intrinsic and extrinsic pathways (Molina-Pinelo *et al.*, 2009; Negredo *et al.*, 2004). In fact, INRs exhibited the most pronounced proliferation of peripheral T cells, reinforcing the direct association with heightened T cell activation and cell death (Anthony *et al.*, 2003; Marchetti *et al.*, 2006). INRs with very poor recovery of $CD4^+$ T cell counts show elevated levels of activation markers ($CD4^+CD38^+CD45RA^-$HLA-DR^+CD95^+), necrosis and overall apoptosis (intrinsic and extrinsic) in $CD4^+$ T cells on day 1, surpassing the rates observed in corresponding concordant individuals that recover $CD4^+$ T cell counts. Levels of $CD4^+$ T-cell apoptosis show a higher correlation than

necrosis levels with the expression of different markers of activation, demonstrating a stronger link between continuous immune activation and apoptosis (Negredo *et al.*, 2010).

An altered gut microbiota can also impede the efficacy of ART and impact the immune reconstitution of patients. The loss of mucosal Th17 cells during HIV infection is another factor leading to microbial translocation, persistent immune activation, and disease progression. The absence of these cells is also linked to the inadequate recovery of $CD4^+$ T cell counts following the initiation of ART. Chronic residual inflammation due to microbial translocation has been linked to suboptimal immune recovery during ART, even when HIV viral suppression is achieved (DaFonseca *et al.*, 2015; Geng *et al.*, 2020).

2 OBJECTIVES AND GENERAL HYPOTHESIS

Chronic infections, such as HIV, can lead to persistent immune activation dysfunction, and exhaustion of $CD4^+$ T cells. This dysfunction is characterized by impaired cytokine production, reduced proliferation, and increased susceptibility to cell death, contributing to the persistence of the infection (Virgin *et al.*, 2009).

The most striking features of HIV infection is the loss of $CD4^+$ T cells. The deficiencies in T cells can be attributed to the specific reduction in the quantity and quality of memory cells. The virus selectively and effectively attacks HIV-specific $CD4^+$ T cells and memory cells located within mucosal tissues (Meyaard *et al.*, 1994). Altered T-cell homeostasis during HIV-1 infection is due to changes in the functional level of T cell memory, which is critical for T cell turnover and T cell depletion in HIV-1 infection (Margolick *et al.*, 1997).

Memory $CD4^+$ T cells are affected during all phases of infection, even after receiving ART. During the initial phases of HIV infection, widespread infiltration of memory $CD4^+$ T cells results in a notable depletion across multiple tissues, nearly half of the body's memory T cells can succumb, but virus-specific cells maintain the ability to develop memory (Johnson *et al.*, 2005). Then, as infection progresses, depletion increases in the memory cell population rather than in fully mature effector cells. The prevalence of cellular exhaustion and the decrease in memory potential increase the deterioration of the immune response (Angelosanto *et al.*, 2012).

The gradual reduction of peripheral blood $CD4^+$ T cells is related to the elevated cell death and the relatively low rate of homeostatic proliferation or immune activation. The continuous cycle of $CD4^+$ T cell activation and proliferation, which favors HIV replication, cannot fully explain the rate of $CD4^+$ T cell death observed in chronic HIV infection (Yates *et al.*, 2007). That is why it is unlikely that the decline in memory $CD4^+$ T cells in HIV-1 infection is due solely to the direct damaging effects of the virus or the elimination of infected cells by CTLs and is rather an increased susceptibility to cell death in uninfected cells (Esser *et al.*, 2001).

PLWH continue to show low levels of chronic inflammation and their $CD4^+$ T cells remain susceptible to cell death even after the introduction of effective ART and complete suppression of plasma HIV RNA levels (Dyrhol-Riise *et al.*, 2001; van Grevenynghe *et al.*, 2008b).

In the search for important molecules and pathways that are involved in the maintenance of $CD4^+$ T cell responses during chronic infections, our laboratory has reported that the upregulation of the transcription factor IRF-5 hindered the survival of IFN-γ^+ $CD4^+$ T cells during the chronic stage

of experimental visceral leishmaniasis. Indeed, activation of IRF-5 via TLR7 by inflammatory tissue damage-derived apoptotic cell material induced the expression of caspase 8 and DR5, and ultimately led IFN-ɣ$^{+}$ CD4^{+} T cells to cell death (Fabié *et al.*, 2018). These findings suggested that cell death of effector CD4^{+} T cells was not promoted by the parasite, but was provoked by the inflammatory environment and the consequent tissue damage. Because tissue disruption and the release of DAMPs are common characteristics of persistent infections, we were wondering whether IRF-5 was involved in cell death during HIV infection.

Similarly to VL, HIV-1 infection is characterized by a significant loss of CD4^{+} T cells, an exacerbated inflammatory milieu, tissue damage, and increased TLR7 expression on CD4^{+} T cells. This led us to hypothesize that IRF-5 may contribute to altered memory CD4^{+} T cell homeostasis during HIV-1 infection and that TLR7 could be upstream of IRF-5.

Hence, the main objective of this work was to elucidate the role of IRF-5 in CD4^{+} T cell maintenance in the context of HIV infection and identify potential IRF-5 signalling partners involved in the induction of cell death.

To this end, the project was structured into the following three specific aims:

1) To determine whether IRF-5 is expressed and is active in human CD4^{+} T cells.

To date, it is not known whether IRF-5 is expressed in human CD4^{+} T cells. To analyze IRF-5 expression and activation in various T cell subpopulations, Peripheral blood mononuclear cells (PBMCs) and CD4^{+} T cell isolated from PLWH and HIV^{free} donors were be used for analysis *ex vivo* of IRF-5 expression and activation in naive population, central memory and effector memory populations by flow cytometry and ImageStream.

2) To identify IRF-5 signaling partners.

We first investigated IRF-5 upstream signaling partners in memory CD4^{+} T cells. Various candidates that have been reported to induce IRF-5 expression in other cell types were considered: e.g., TLR7, type I IFN receptor, cytosolic receptors (RIG-I), and TCR signaling (Fabié *et al.*, 2018; Lazear *et al.*, 2013; Yan *et al.*, 2020). Since we had previously shown that TLR7 was upstream of IRF-5 on CD4^{+} T cells and during chronic murine *L. donovani* infection (Fabié *et al.*, 2018), we hypothesized that this endosomal receptor could induce IRF-5 expression during HIV infection. To determine this, we first analyzed whether TLR7 is differentially expressed in CD4^{+} T cells from PLWH. To this end, the gene expression of this receptor was analyzed by qPCR. In

addition, To confirm the signaling pathway upstream of IRF-5 activation, purified $CD4^+$ T cells were treated *in vitro* with Imiquimod (TLR7 agonist), anti-CD3/anti-CD28 (TCR-stimulation), IFNα/β, RIG-I agonist, and/or apoptotic cell material. ImageStream was used to determinate the activation status of IRF-5.

To determine the downstream IRF-5 signaling partners, the expression of DR5, FAS, TNFR1, and annexin V /caspase-3 (cell death) were analyzed by flow cytometry in various $CD4^+$ T cells subpopulations, and the expression of *CASP8* and cell-cycle regulators were assessed by qPCR.

If TLR7 is upstream of IRF-5, we also investigated possible pathways that lead to the upregulation of TLR7 in $CD4^+$ T cells. We initially tested TCR simulation, DAMPs sensing, and IFN-I exposure, three stimuli that are typically present during infections.

3) To investigate whether IRF-5–induced cell death could be blocked.

Finally, if our hypothesis is correct and IRF-5 promotes cell death in the memory $CD4^+$ T cell compartment, we sought to block IRF-5 activation to limit cell death. To do this, we tested cell-permeable IRF-5 inhibitory peptides (IRF-5-CPPs) that were recently published by Banga et al. (Banga *et al.*, 2020), alone or in combination. Levels of apoptosis and cell death were determined using Zombie Aqua Fixable Viability Kit, detection of caspase 3, and annexin V by flow cytometry.

The results of this study are detailed in Chapter 3.

3 ARTICLE

The TLR7-IRF-5 axis sensitizes memory CD4$^+$ T cells to Fas-mediated apoptosis during HIV-1 infection

Titre de l'article en français: L'axe TLR7-IRF-5 sensibilise les cellules T CD4$^+$ mémoires à l'apoptose médiée par Fas au cours de l'infection par le VIH-1.

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Contribution des auteurs: LCP and S. Stäger conceived the project, designed the experimental approach, interpreted data, and wrote the manuscript with comments from all authors; LCP performed experiments and analyzed data; XDL, LTM, TS, and S. Swaminathan performed experiments; MRR, SI, and BJB provided key reagents and expertise; and JVG and JPR provided key expertise, interpreted data, and revised the manuscript.

3.1 Abstract

HIV-1 infection is characterized by a strong inflammatory environment, tissue disruption, and a progressive decline in CD4+ T cell count. Despite treatment with antiretroviral therapy (ART), the majority of persons living with HIV (PLWH) maintain residual levels of inflammation, low degree of immune activation, and higher sensitivity to cell death in their memory CD4+ T-cell compartment. To date, the mechanisms responsible for this high sensitivity remain elusive. We have identified the transcription factor IRF-5 to be involved in impairing the maintenance of murine CD4+ T cells in a chronic inflammatory environment. Here, we investigate whether IRF-5 also contributes to memory CD4+ T cell loss during HIV-1 infection.

We show that TLR7 and IRF-5 were upregulated in memory CD4+ T cells from PLWH, when compared with naturally protected elite controllers and HIV^{free} participants. TLR7 was upstream of IRF-5, promoting Caspase 8 expression in CD4+ T cells from ART HIV-1+ but not from HIV^{free} participants. Moreover, IRF-5 and TLR7 expression inversely correlated with CD4+ T cell counts in primary HIV infection. Interestingly, the TLR7-IRF-5 axis acted synergistically with the Fas/FasL pathway, suggesting that TLR7 and IRF-5 expression in ART HIV-1+ memory CD4+ T cells represents an imprint that predisposes cells to Fas-mediated apoptosis. This predisposition could be blocked using IRF-5 inhibitory peptides. Thus, we propose IRF-5 blockade as a possible therapy to prevent memory CD4+ T cell loss in PLWH.

Keywords: AIDS/HIV, Immunology, Apoptosis, T cells

3.2 Introduction

Infections with the Human Immunodeficiency Virus type 1 (HIV-1) remains a major public health issue. Unlike a number of other viruses, the human body cannot clear HIV, even with ART, making it a life-long infection (Chun *et al.*, 1997; Finzi *et al.*, 1997; Wong *et al.*, 1997).

One of the hallmarks of HIV infection is the progressive loss of $CD4^+$ T cells, which affects both infected and bystander cells. $CD4^+$ T cell death occurs via several mechanisms, including direct viral cytopathogenicity, killing by $CD8^+$ cytotoxic T lymphocytes, and pyroptosis in infected $CD4^+$ T cells, or apoptosis in uninfected bystander cells, with the latter being largely Fas/FasL-mediated (Badley *et al.*, 1998; Doitsh *et al.*, 2014; Katsikis *et al.*, 1995; Massanella *et al.*, 2013; Monroe *et al.*, 2014). Maintenance of a critical count of $CD4^+$ T cells is essential to prevent secondary opportunistic infections. ART efficiently controls viral replication and the majority of PLWH are able to restore $CD4^+$ T cell count with therapy. Nevertheless, it is still unclear why $CD4^+$ T cell homeostasis is not fully normalized in PLWH on ART. More importantly, memory $CD4^+$ T cells in those PLWH are more prone to Fas-mediated apoptosis (Dyrhol-Riise *et al.*, 2001; van Grevenynghe *et al.*, 2008b). The mechanisms leading to this heightened sensitivity are only partially known and involve the proapoptotic activity of the transcription factor Foxo3a (van Grevenynghe *et al.*, 2008b). Although viral replication is under control, residual levels of immune activation and inflammation persist in infected individuals on ART(Finzi *et al.*, 1999; Wada *et al.*, 2015).

In the search for molecules and pathways involved in the maintenance of $CD4^+$ T cells in chronic inflammatory environments, we have recently identified the transcription factor interferon regulatory factor 5 (IRF-5) as a detrimental player in the survival of $CD4^+$ T cells during chronic visceral leishmaniasis (Fabié *et al.*, 2018). IRF-5 is a member of the interferon regulatory factor (IRF) family, a group of transcription factors with diverse roles, including transcriptional activation of interferons, and modulation of cell growth, differentiation, apoptosis, and immune system activity (Honda *et al.*, 2006b; Tamura *et al.*, 2008). In recent years, IRF-5 has gained much attention for its role in regulating inflammatory responses in immunological diseases. IRF-5 is constitutively expressed in antigen presenting cells, where it is involved in the activation of inflammatory cytokines and type I interferon (IFN-I) production (Barnes *et al.*, 2001; Barnes *et al.*, 2004; Honda *et al.*, 2006b; Takaoka *et al.*, 2005) and has been identified as a marker for M1 macrophage polarization (Krausgruber *et al.*, 2011). Moreover, IRF-5 acts as a tumor suppressor (Hu *et al.*, 2005) and is critical for the induction of apoptosis in response to DNA damage in tumor cells (Bi *et al.*, 2014a). IRF-5 also mediates apoptosis by modulation of proapoptotic genes

including DR5, Fas, Caspase 8, DAP kinase 2, Bak, and Bax. (Hu *et al.*, 2009). In B cells, IRF-5 induces activation, proliferation, and plasmablast differentiation (De *et al.*, 2017) and is required for antibody production and class switching (Fang *et al.*, 2012). However, while IRF-5 appears to be required for the optimal assembly of the TCR-initiated signaling complex (Yan *et al.*, 2020), its role in T cells is not yet completely understood

We have also shown that IRF-5 was increasingly expressed during chronic *Leishmania donovani* infection in mice and could be activated via TLR7 expressed in T cells. Interestingly, IFNγ$^+$ CD4$^+$ T cells were capable of sensing tissue damage-derived apoptotic cell material via TLR7; activation via TLR7 by apoptotic cell material induced DR5 (or TRAILR2) expression and ultimately led to cell death (Fabié *et al.*, 2018). These findings suggest that sensing of damage-associated molecular patterns (DAMPs) released following tissue damage in a chronic inflammatory environment and the concomitant TLR7 upregulation by CD4 T cells can promote cell death. As HIV-1 infection is characterized by a strong inflammatory environment, tissue disruption, upregulation of TLR7 in CD4$^+$ T cells, and a progressive decline in their function (Dominguez-Villar *et al.*, 2015; McCune, 2001a; Moir *et al.*, 2011; Schacker *et al.*, 2002), we were wondering whether the TLR7-IRF5 pathway was involved in impairing human memory CD4$^+$ T cell homeostasis during HIV-1 infection.

Herein, we showed that IRF-5 displayed higher levels of expression and activation and TLR7 was upregulated in CD4$^+$ T cells from PLWH when compared with elite controllers (EC) and HIV^{free} participants. Moreover, triggering of TLR7 promoted IRF-5 expression, which in turn induced Caspase 8 expression in CD4$^+$ T cells from ART HIV-1$^+$ but not from HIV^{free} individuals, sensitizing these cells to Fas-mediated apoptosis. This cell death pathway could be blocked with cell penetrating, IRF-5 blocking peptides. Hence, we propose that heightened expression of TLR7 and IRF-5 in memory CD4$^+$ T cells from PLWH on ART represents an imprint that affects memory CD4$^+$ T cell homeostasis.

3.3 Materials and Methods

3.3.1 Study population

Participants were selected from the Montreal Primary HIV infection study that received approval from the McGill University Health Centre Ethical Review Board and at each study sites (ethic reference number SL-00.069), Montreal QC, Canada and were middle-aged subjects (range: 27–56 years old). Age-matched uninfected participants were selected as a negative control for HIV-

1 infection from the Investigating IRF-5 signaling pathway in $CD4^+$ T cell study (ethic reference number CER-21-599) that received approval from Ethics Committee for Research of Institut national de la recherche scientifique (INRS) Laval, QC, Canada. All subjects provided informed and written consent for participation. PLWH included in the study were homogeneously selected and displayed similar clinical and virological data. PHI participants, whose viral acquisition was estimated to have occurred less than four months prior to sample collection, displayed viral loads ranging from 3.08 $x10^3$ to 513.54 $x10^3$ copies/ml. PLWH on ART displayed both viral suppression (viral loads < 40 copies/ml) and $CD4^+$ T cell recovery (> 400 cells/µl in blood post-treatment) for at least 5 years. EC participants were selected displaying a long-term viral control (undetectable viral loads < 40 copies/ml) without treatment for more than 5 years, and did not have protective gene polymorphisms such as CCR5 Δ32 and B57/B27 haplotypes, with $CD4^+$ T cell counts over 400 cells/µl in their blood. Demographic and clincal characteristics of each study group are summarized in Table 3.1.

Table 3.1 Characteristics and clinical data of all HIV-1^+ and HIV^{free} individuals

Characteristics	HIV^{free}	PHI	ART HIV-1^+	EC
N	17	10	17	9
Sex F/M	7/17	1/9	7/17	1/8
Age (years) *	35 (28-55)	33 (28-53)	42 (29-56)	35 (27–51)
Time of infection*	N/A	63 (32-119) days	> 3 years	> 3 years
CD4 count, cells/ µl*	1028 (1288-757)	585 (420-1000)	583 (450-820)	753 (635-753)
Viral load (copies/ml) *	N/A	$40.57x10^3$ ($3.08\text{-}513.54\ x10^3$)	<40	<40

3.3.2 Cell culture reagents

Cells were cultured in RPMI 1640 media (Gibco; Invitrogen) supplemented with 3.2 % L-Glutamine (Cellgro), 10 ml of 2-Mercaptoethanol 55mM (Gibco), 50% Sodium Pyruvate 100mM (Gibco) 10% penicillin/streptomycin (Gibco, Invitrogen) and 10% fetal bovine serum (Wisent). The antiviral Azidothymidine (AZT Sigma Aldrich; 10µM) was added to all cultures from HIV-1$^+$ individuals to prevent viral replication and de novo infections, as previously reported (Dagenais-Lussier *et al.*, 2016). Cells were washed with Dulbecco's Phosphate-buffered saline (DPBS Gibco, Invitrogen) before RNA extraction or immunofluorescence staining for flow cytometry. From each experimental condition, cells and cell lysates were prepared for the different analyses described below.

3.3.3 CD4$^+$ T cell purification

PBMCs were thawed, resuspended in 1ml of FBS, and centrifuged for 5 minutes at 1400 rpm. The supernatant was removed, and the cells were resuspended in 3ml of RPMI medium with 10% FBS and incubated at 37◦ C for 2h before the CD4$^+$ T cell purification.

CD4$^+$ T cells from ART HIV-1$^+$, EC, and HIV^{free} samples were isolated from PBMCs using the EasySep human CD4$^+$ T cell enrichment kit (StemCell Technologies) following manufacturer's instructions, allowing for more than 92% purity as determined by flow cytometry.

3.3.4 *In vitro* CD4$^+$ T cell stimulation

1 x 10^6 CD4$^+$ T cells were activated for 24 h with 1 µg/ml of anti-CD3 (clone OKT3, eBioscience) and 1 µg/ml of anti-CD28 Abs (clone CD28.2, Invitrogen and eBioscience). To assess TLR7 agonist effect on CD4$^+$ T cells, 1 x 10^6 cells/ml were stimulated with 5 µg/ml of imiquimod (Invivogen) for 30 h. For IFN-β stimulation, CD4$^+$ T cells were cultured with 10 ng/ml IFN-β (Peprotech) for 6 h and washed three times with RPMI. For some assays, 1 µg/ml of Brefeldin A (GolgiPlug^{TM}, BD Biosciences) was added to cell cultures for 6 h to evaluate the intracellular accumulation of inflammatory cytokines.

To investigate possible pathways resulting in TLR7 expression in CD4$^+$ T cells during HIV-1 infection, we pre-treated the 1 x 10^6 CD4$^+$ T cells from HIV^{free} donors with 10 ng/ml IFN-β for 6 h

in the presence or absence of conditioned medium with 10% v/v of DAMPs containing supernatant, obtained from PBMCs treated with staurosporine for 24 h, before TCR activation or TLR7 stimulation. The supernatant was prepared as follows: PBMCs from HIV^{free} donors were cultured *in vitro* at a concentration of 10^6/ml, in the presence of 2 µM staurosporine for 2h at 37C; cells were then washed three times with sterile PBS and left in culture for further 4h at 37C, before collecting the supernatant.

3.3.5 Fas-induced apoptosis and cell death

Purified $CD4^+$ T cells were cultured in the presence or absence of 10 ng/ml of recombinant human Fas ligand (rFasL or TNFSF6, Biolegend) for 18 h. Apoptosis detection was performed using rabbit anti-h cleaved Caspase-3 (Asp175)-PE (clone D3E9, Cell signaling), AnnexinV-APC (BD or Biolegend) and Zombie Aqua™ Fixable Viability Kit (Biolegend) labeling according to the manufacturer's protocol and subsequent analysis by flow cytometry. The percentage of Fas-induced apoptosis was determined by the formula: % of apoptosis in the presence of rFasL – % of apoptosis in absence of rFasL, as previously described (van Grevenynghe *et al.*, 2008b).

3.3.6 $CD4^+$ T cells and PBMCs stimulation in the presence of inhibitory peptides

We first analyzed the cytotoxicity of different IRF-5-cell permeable peptides (IRF-5-CPPs), described in Table 3.2, on PBMCs and isolated $CD4^+$ T cells. Cells were incubated with various concentrations of IRF-5-CPPs (5, 20, and 50µM) for 24h. We evaluated cell death by flowcytometry and determined IRF-5 nuclear localization by ImageStream X MKII. To further confirm the specificity of IRF-5-CPPs, we used a scrambled version of the peptides as negative control and analyzed their ability to inhibit IRF-5 homodimerization and activation after IMQ treatment by ImageStream X MKII. We used the combination of IRF-5-CPPs (P1 and P3) that could inhibit the nuclear localization of human IRF-5 in $CD4^+$ T cells for the rest of the experiments. $CD4^+$ T cells were pretreated with 10 µM of IRF-5-CPPs for 30 min before stimulation with 5 ng/ml IMQ for 30h at 37°C; or pretreated cells were stimulated with 5 ng/ml IMQ and, 12h later, 10 ng/ml rFasL was added to the culture for further 18h of incubation.

Table 3.2 IRF-5-cell permeable peptides sequences

Peptide	Sequence	Modification	Purity
Peptide 1	MIILIISFPKHKDWKVILVK	N-Terminal: Acetylation, C-Terminal: Amidation, TFA removal (HCl)	>95%
Peptide 2	IRLQISNPYLKFIPLKRAIWLIK	N-Terminal: Acetylation, C-Terminal: Amidation, TFA removal (HCl)	>95%
Peptide 3	MANLGYWLLALFVTYWTDLGLVKKRPKP	TFA removal (HCl)	>95%
Peptide 4	MANLGYWLLLLFVTMWTDVGLAKKRPKP	TFA removal (HCl)	>95%
Control Peptide	IKVMWPILFIIKLVHSDKKI and MANLGYWLLALFVTMWTDVGLCKKRPKP	TFA removal (HCl) Scrambled	>95%

3.3.7 Flow cytometry

Purified $CD4^+$ T cells and PBMCs were labeled with the fluorochrome-conjugated monoclonal antibodies described below. Mouse anti-hCD3-BV711 (clone UCHT1, BD or Biolegend), mouse anti-hCD4-Alexa flour 700 (clone RPA-T4 , BD or Biolegend), mouse anti-hCD45RA-APC-H7 (clone HI100, BD), mouse anti-hCD45RA-APC-Cy7 (clone HI100, Biolegend), mouse anti-hCD27-PerCP-Cy5.5 (clone RPA-T4 , BD or Biolegend), mouse anti-hCD197 (CCR7)-BV605 (clone 2-L1-A, BD), mouse anti-hCD197 (CCR7)-BV605 (clone G043H7, Biolegend), mouse anti-hIFNγ-PE-Cy7 (clone 4S.B3, BD), mouse anti-hDR5 (CD262) (clone B-K29, BD), mouse anti-hCD95-FITC (clone DX2, BD), mouse anti-hCD95-PE (clone DX2, BD), mouse anti-hCD95-PE-CF594 (clone DX2, BD), rabbit anti-hIRF-5-PE (clone E1N9G, Cell signaling, discontinued), sheep anti-hIRF-5-Alexa Fluor488 (R&D systems), rabbit anti-h cleaved Caspase-3 (Asp175)-PE (clone D3E9, Cell signaling), AnnexinV-APC (BD and Biolegend), mouse anti-hIgG1, κ isotype control PE-CF594 (clone X40, BD), mouse anti-hIgG1 κ isotype control- PE-Cy7 (clone MOPC-21, BD), rabbit anti-hIgG XP-PE isotype control (clone DA1E, Cell signaling), sheep anti-hIgG isotype control-Alexa Fluor488 (R&D systems), and Zombie Aqua™ Fixable Viability Kit

(Biolegend). Cells were fixed with 4% Paraformaldehyde (PFA) for 10 minutes at room temperature. The cellular permeabilization was done using 0.25% (W/V) saponin in PBS, following intracellular staining assays using rabbit anti-hIRF-5-PE (clone E1N9G, Cell signaling), sheep antihIRF-5-Alexa Fluor488 (R&D systems), rabbit anti-hcleaved Caspase-3 (Asp175)-PE (clone D3E9, Cell signaling), mouse anti-hIgG1, κ isotype control PE-CF594 (clone X40, BD), mouse anti-hIgG1 κ isotype control- PE-Cy7 (clone MOPC-21, BD), rabbit anti-hIgG XP-PE isotype control (clone DA1E, Cell signaling) and sheep anti-hIgG isotype control-Alexa Fluor488 (R&D systems). Flow cytometry analysis was performed with a BD LSRII flow cytometer (BD Biosciences). Data were analyzed with Flowjo and DIVA software (BD Biosciences). 200,000–500,000 gated cells were analyzed for each sample.

3.3.8 ImageStreamX flow cytometry

Samples were stained with mouse anti-hCD3-BV711 (clone UCHT1, BD and Biolegend), mouse anti-hCD4-Alexa flour 700 (clone RPA-T4, BD and Biolegend), mouse anti-hCD8-BV650 (clone RPA-T8, BD), mouse anti-hCD19-V450 (clone HIB19, BD), mouse anti-hCD14- PE-Cy7 (clone M5E2, BD). The cells were then fixed, permeabilized, and stained with DAPI (Invitrogen) to visualize the nucleus and mouse anti-hIFNγ-PE-Cy7 (clone 4S.B3, BD), along with mouse anti-hIRF-5- PE (Biolegend), rabbit anti-hIRF-5-PE (clone E1N9G, Cell signaling), or sheep anti-hIRF-5-Alexa Fluor488 (R&D systems). Finally, samples were acquired using the ImageStreamX MKII flow cytometer and analyzed with IDEAS software (Amnis, Seattle, WA, USA). The localization wizard was used to determine the nuclear localization of IRF-5. The morphology mask was used to determine a similarity score, which quantifies the correlation of pixel values of the DAPI and IRF-5 images on a per cell basis. A similarity score >1 was used as a cut-off for nuclear localization. Cells in individual bins were visually inspected to confirm subcellular localization (values < or >1). 200,000–500,000 gated cells were analyzed for each sample.

3.3.9 RNA isolation and quantitative real-time PCR analysis

RNeasy mini kit (QIAGEN) was used for RNA isolation. RNA yield and purity were measured by Nanodrop (Thermo Scientific). RNA isolated from $CD4^+$ T cells and PBMCs cells were used to generate cDNAs by reverse transcription using the iScript cDNA synthesis kit (Bio-Rad). cDNAs

(Livak *et al.*, 2001; Schmittgen *et al.*, 2008)using iTaq Universal SYBR Green Supermix (Bio-Rad) and specific primers for human *IRF5*, *TLR7*, *CASP8*, and *GAPDH* genes, which are summarized in Table 3.3. The transcription level of GAPDH was used as housekeeping gene to normalize the expression levels of target genes. Relative gene expression of target genes in each sample was determined by the comparative CT method ($2^{-\Delta\Delta ct}$) (Livak *et al.*, 2001; Schmittgen *et al.*, 2008).

Table 3.3 Primers used for RT-PCR

Gene name	Forward	Reverse
GAPDH	5′-GAAGGTGAAGGTCGGAGTC-3′	5′-GAAGATGGTGATGGGATTTC-3′
IRF5	5′-TTATTCTGCATCCCCTGGAG-3′	5′-GCTCTTGTTAAGGGCACAGC-3′
TLR7	5'-TTTACCTGGATGGAAACCAGCTA-3'	5'-TCAAGGCCTGAGAAGCTGTAAGCTA-3'
CASP8	5'-AGAGTCTGTGCCCAAATC AAC -3'	5'-GCTGCTTCTCTCTTTGCTGAA -3'

3.3.10 Generation of IRF5 knockout THP-1 cells

The sgRNA for IRF-5 was obtained from IDT (target sequence 5' GTA CTG GCA GCT GTT CAC CT). Recombinant Alt-R S.p. Cas9 nuclease (85 µg) was incubated with each target sgRNA (6 µL of 100 µM stock) for 20 min at RT. The reaction was supplemented with Alt-R Cas9 Electroporation Enhancer (4 µL) and combined with 4 x 10^6 cells resuspended in complete P3 nucleofection solution (71µL) (Lonza). The samples were subjected to nucleofection using program CM-137 and maintained in RPMI. At 5 days post-nucleofection, 3 x 10^6 cells were collected to confirm knockout efficiency by immunoblot analysis.

3.3.11 Statistical analysis

Parameters of patients and controls are described as absolute frequencies in the case of qualitative variables (gender); as medians and interquartile ranges for non-normally distributed quantitative variables (age, viral loads, CD4 counts); and as means and standard deviations (SD) for normally distributed quantitative variables. Comparison of quantitative variables of each study

group of subjects was performed using Kruskal-Wallis test, followed by the Dunn's multiple comparison test. Frequencies, median fluorescence intensity, and cell numbers positive for each marker were compared with the Mann-Whitney U test. Variables of *in vitro* experiments performed with and without were compared with the Wilcoxon test and Dunn's multiple comparison test. Correlation analyses were performed with Pearson's or Spearman's coefficients. In all cases, a p-value ≤ 0.05 was considered statistically significant. Statistical analyses were run in GraphPad Prism version 6 (GraphPad Software; La Jolla, CA).

3.4 Results

3.4.1 IRF-5 is highly expressed and active in $CD4^+$ T cells from primary HIV-infected donors (PHI)

We have previously reported that activation of the transcription factor IRF-5 in murine $IFNγ^+$ $CD4^+$ T cells leads to cell death during chronic visceral leishmaniasis (Fabié *et al.*, 2018). Interestingly, IRF-5 was induced following TLR7 triggering by apoptotic cell material derived from inflammatory tissue disruption, suggesting that damage-associated molecular pattern (DAMP)-sensing by $CD4^+$ T cells may not be microbial-specific. Hence, we were wondering whether IRF-5 is also expressed and promotes cell death in human $CD4^+$ T cells in the context of HIV infection. We chose to analyse the function of IRF-5 in $CD4^+$ T cells from PLWH as the infection is associated with a strong inflammatory environment and with a loss of $CD4^+$ T cells before ART initiation (PHI phase), and because TLR7 is expressed on $CD4^+$ T cells during HIV-1 infection (Dominguez-Villar *et al.*, 2015). PHI participants were between 27 and 56 years old, displayed viral loads ranging from 3.08 $x10^3$ to 513.54 $x10^3$ copies/ml, and their viral acquisition was estimated to have occurred less than four months prior to sample collection. Age-matched uninfected participants were selected as a negative control for HIV-1 infection. The demographic and clinical characteristics of each group are summarized in Table 3.1.

Before we proceeded to analyse IRF-5 expression in $CD4^+$ T cells from PHI participants, we tested the anti-human IRF-5 antibody from R&D Systems for its specificity, which has been a problem for many commercial antibodies recognizing IRF-5 (Li *et al.*, 2016b). To this end, we activated IRF-5-sufficient and -deficient THP-1 cells with imiquimod (IMQ) or PMA plus IMQ. More than half of the unstimulated THP-I cells constitutively expressed IRF-5; this percentage increased upon stimulation with IMQ and nearly all the cells expressed IRF-5 after incubation with PMA + IMQ (supplemental Figure 3.10A). In contrast, we did not detect IRF-5 expression in *Irf5*$^{-/-}$ THP-I cells

even after stimulation with IMQ or PMA+IMQ (supplemental Fig.1A), suggesting that this anti-human IRF-5 antibody is specific. We also determined IRF-5 nuclear localization by ImageStreamX (supplemental Fig.1B).

We found a higher frequency of IRF-5 expressing $CD4^+$ T cells in PHI participants when compared with $CD4^+$ T cells from HIV^{free} control participants (Fig. 1A; gating strategy for analysis of PHI $CD4^+$ T cells is shown in supplemental Fig.1C). Moreover, this transcription factor co-localized with the nucleus in about 40-60% of IRF-5^+ $CD4^+$ T cells from HIV-1^+ donors compared to 1.8-6.7% in $CD4^+$ T cells from HIV^{free} control participants (Fig. 1B and C). Similarly to what we previously observed in mice (Fabié *et al.*, 2018), the majority of cells expressing IRF-5 were also secreting IFN-ɣ (Fig. 1D) and were mainly effector and memory cells (Fig. 1E and supplemental Fig.2A). As expected, $CD4^+$ T cells from PLWH also showed higher frequencies of $AnnexinV^+$ cells, a common marker of cell death and apoptosis, compared with cells from HIV^{free} control participants (Fig. 1F). Of note, IRF-5 expression positively correlated with AnnexinV expression (Fig. 1G) and AnnexinV expression inversely correlated with $CD4^+$ T cell count in PLWH (Fig.1H). Moreover, IRF-5 expression also inversely correlated with $CD4^+$ T cell count (Fig. 1I), suggesting that this transcription factor could contribute to cell death.

In mice, IRF-5 has been shown to promote cell death by inducing the expression of death receptor 5 (DR5 or TRAILR2) (Fabié *et al.*, 2018). However, we did not observe any difference in DR5 expression between $CD4^+$ T cells from PHI and HIV^{free} donors (supplemental Fig. 2B), even though $DR5^+$ cells were mostly IRF-5^+ (supplemental Fig. 2C) and nearly all $IFNɣ^+$ cells were also $DR5^+$ (supplemental Fig. 2D). Moreover, DR5 expression did not correlate with AnnexinV expression (supplemental Fig. 2E), suggesting that, contrary to what observed in *L. donovani* infected mice (Fabié *et al.*, 2018), DR5 is most likely not involved in $CD4^+$ T cell death in PHI individuals.

Figure 3.1

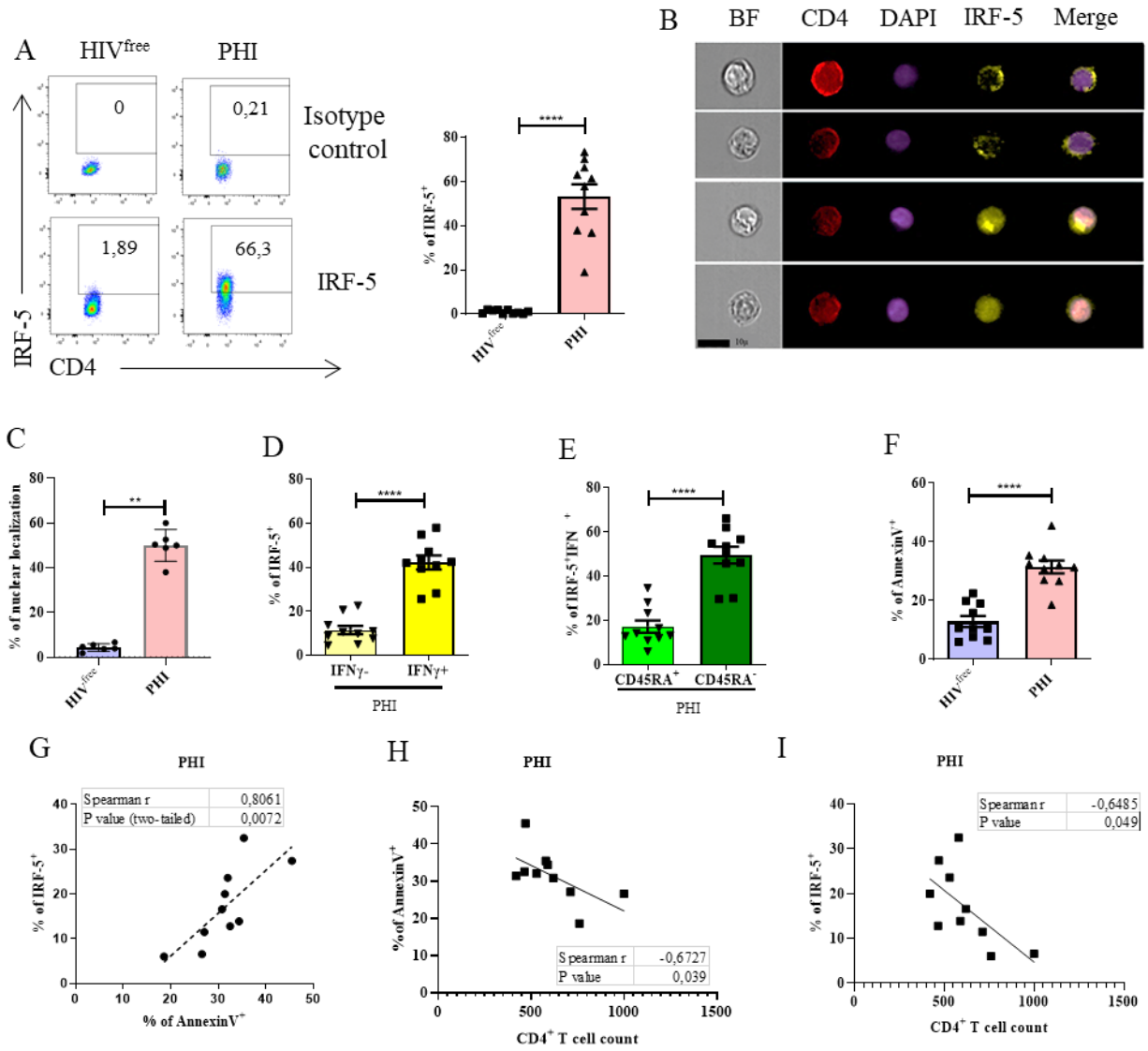


Figure 3.1 IRF-5 is expressed in CD4⁺ T cells from PHI

PBMCs from PHI and HIV^{free} individuals were analyzed ex-vivo by flow cytometry. A) Representative FACS plots and percentage of IRF-5^{+} CD4^{+} T cells. B) Representative ImageStreamX images of CD4^{+} T cells expressing IRF5 in the cytoplasm (first two lanes) and in the nucleus (third and fourth lane). Graphs show C) the frequency of CD4^{+} T cells whose IRF-5 expression co-localizes with the nucleus; D) the percentage of IFNɣ$^{+}$ and IFNɣ$^{-}$ IRF-5^{+} CD4^{+} T cells; E) the frequency of CD45RA^{+} and CD45RA^{-} IFNɣ$^{+}$ CD4^{+} T cells expressing IRF-5 in PHI individuals; and F) the percentage of Annexin V^{+} CD4^{+} T cells from PHI and HIV^{free} individuals. Data are presented as the mean ± SD. The Mann-Whitney *U*-test was used to determine statistical differences, * p <0.05, ** p< 0.01, *** p<0.001, n=10. Graphs represent correlations between G) Annexin V and IRF-5 expression in CD4^{+} T cells, H) Annexin V expression and CD4^{+} T cell

count, and I) IRF-5 expression and CD4$^+$ T cell count in PHI donors. The Spearman r test was used to determine statistical significance, * p <0.05, ** p< 0.01, n=10.

3.4.2 ART HIV-1$^+$ patients show higher frequencies of IRF-5-expressing CD4$^+$ T cells

HIV-1$^+$ patients treated with ART still show low grade inflammation (Finzi *et al.*, 1999; Wada *et al.*, 2015) and their memory CD4$^+$ T cells are prone to Fas-mediated apoptosis despite years of aviremia in the blood compartment (Dyrhol-Riise *et al.*, 2001; van Grevenynghe *et al.*, 2008b). Thus, we next wanted to know whether IRF-5 is also expressed in memory CD4$^+$ T cells from HIV-1$^+$ patients undergoing ART and is involved in sensitizing memory CD4$^+$ T cells to apoptosis.

PLWH on ART that participated in this study displayed both viral suppression (viral loads < 40 copies/ml) and CD4$^+$ T cell recovery (> 400 cells/µl in blood post-treatment) for at least 5 years. Elite controller (EC) participants had undetectable viral loads (< 40 copies/ml) without treatment for more than 5 years, with CD4$^+$ T cell counts over 400 cells/µl in their blood. The demographic and clinical characteristics of each study group are summarized in Table 3.1.

We found significantly higher frequencies of IRF-5-expressing CD4$^+$ T cells in ART HIV-1$^+$ individuals (Fig. 2A) compared to controls. Moreover, IRF-5 co-localized with the nucleus in nearly the half of the IRF-5$^+$ cells (Fig. 2B; gating strategy for analysis of CD4$^+$ T cells from ART$^+$ HIV-1$^+$ donors are shown in supplemental Fig. 3). Since HIV-1$^+$ elite controllers (ECs) can control plasma viral load while maintaining CD4$^+$ T cell count in absence of ART, we were interested in determining the percentage of IRF-5-expressing CD4$^+$ T cells isolated from EC donors. Conversely to ART HIV-1$^+$ individuals, EC presented with lower frequencies of IRF-5$^+$ CD4$^+$ T cells (Fig. 2A), with IRF-5 being active in about 20-25% of the IRF-5$^+$ cells (Fig. 2B). Independently of expression levels, IRF-5 was mostly expressed in effector and memory CD4$^+$ T cells in all three study groups (Fig. 2C). As IRF-5 is expressed in memory CD4$^+$ T cells and these cells are more prone to apoptosis in ART HIV-1$^+$ patients, we decided to focus on memory CD4$^+$ T cells. Interestingly, memory CD4$^+$ T cells from ART HIV-1$^+$ but not EC donors expressed significantly higher levels of AnnexinV (Fig.2D) when compared with the uninfected control group, suggesting that memory CD4$^+$ T cell death was more frequent in ART HIV-1$^+$ patients. Expression of AnnexinV in memory CD4$^+$ T cells correlated with IRF-5 expression (Fig 2E), suggesting that this transcription factor may contribute to memory cell death in ART HIV-1$^+$ donors. No correlation was found between IRF-5 and Annexin V expression in memory cells from ECs (supplemental Fig. 4A).

Figure 3.2

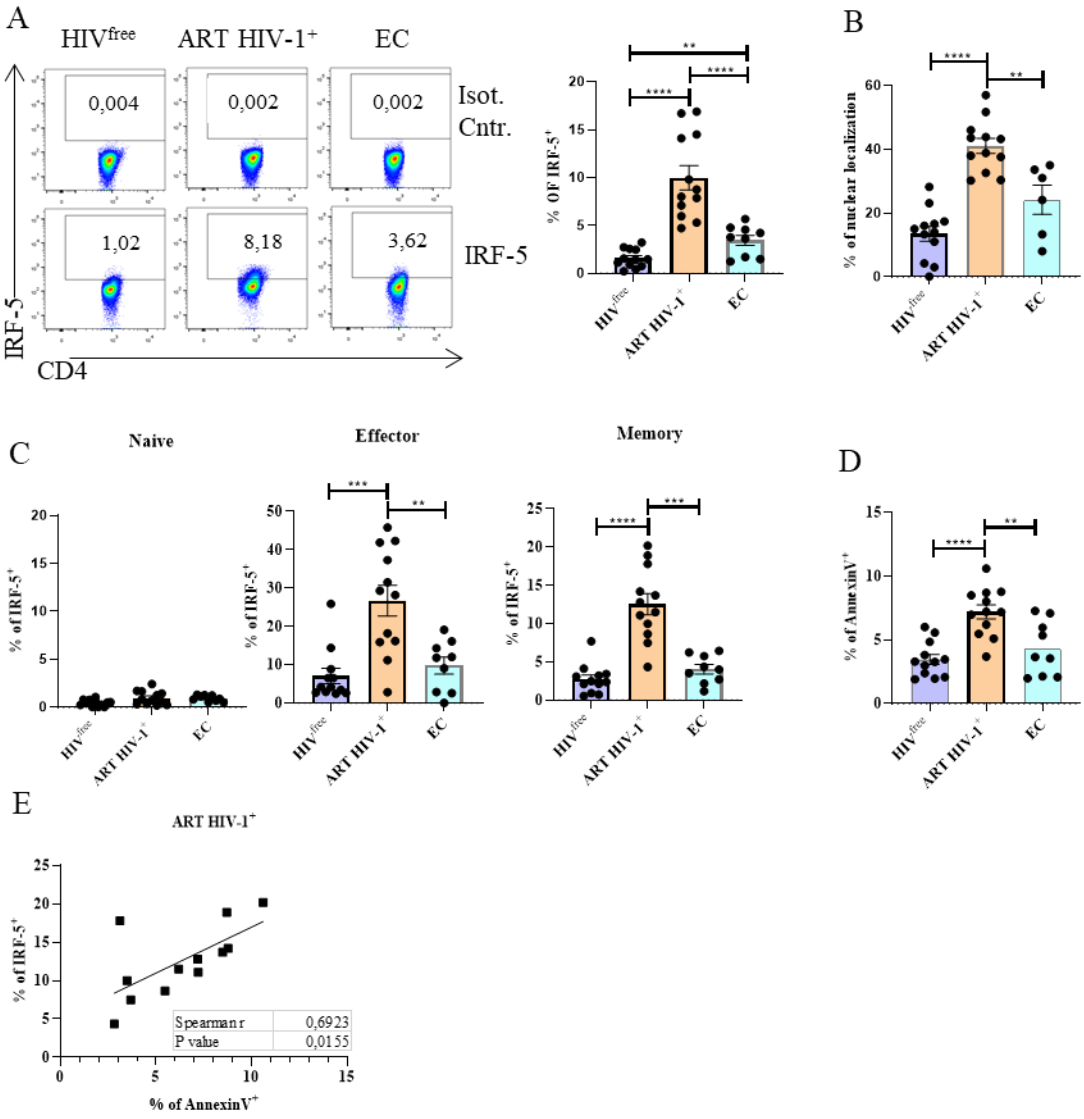


Figure 3.2 IRF-5 is expressed in memory and effector CD4⁺ T cells from ART HIV⁺

PBMCs from ART HIV-1⁺, EC, and HIV^{free} donors were analyzed ex-vivo by flow cytometry. A) Representative FACS plots and percentage of IRF-5⁺ CD4⁺ T cells. Graphs show B) the percentage of CD4⁺ T cells expressing IRF-5 in the nucleus; C) the percentage of IRF-5 expression in naïve, effector and memory CD4 ⁺ T cell; and D) the percentage of memory CD4⁺ T cells positive for Annexin V in PBMCs isolated from ART HIV-1⁺, EC, and HIV^{free} individuals. Data are presented as the mean ± SD. The Mann-Whitney *U*-test was used to determine statistical differences, * p <0.05, ** p< 0.01, *** p<0.001, n=12 (ART HIV-1⁺ and HIV^{free}), n=9 (EC). E) Graph represents correlation between Annexin V and

IRF-5 expression in memory CD4$^+$ T cells from ART HIV-1$^+$ patients. The Spearman r test was used to determine statistical significance, * p <0.05, n=12.

3.4.3 TLR7 is upstream of IRF-5 in memory CD4$^+$ T cells from ART HIV-1$^+$ individuals

We next wanted to investigate IRF-5 upstream signalling partners in memory CD4$^+$ T cells. We have previously demonstrated that TLR7 was increasingly expressed in CD4$^+$ T cells over the course of *Leishmania donovani* infection in mice and that TLR7 triggering resulted in IRF-5 induction (Fabié *et al.*, 2018). TLR7 is known to be expressed in human CD4$^+$ T cells during HIV-1 infection (Dominguez-Villar *et al.*, 2015), but it is still unknown whether TLR7 expression is maintained in CD4$^+$ T cells after ART. Hence, we analyzed *TLR7* mRNA expression in CD4$^+$ T cells isolated from ART HIV-1$^+$, EC, and HIV^{free} individuals. The highest levels of *TLR7* mRNA expression were detected in CD4$^+$ T cells from ART HIV-1$^+$ donors, while ECs expressed intermediate *TLR7* levels compared with HIV^{free} donors (Fig. 3A). Subsequently, we stimulated CD4$^+$ T cells from the different groups of study with imiquimod (IMQ), a TLR7 agonist. We found that treatment of the cells with IMQ induced IRF-5 expression in memory CD4$^+$ T cells from ART HIV-1$^+$ and to a lesser extent from EC donors, but not in HIV^{free} individuals (Figure 3B). To understand what would trigger TLR7 and activate IRF-5 in vivo, we measured regenerating islet-derived protein 3 alpha (REG3α) in the serum of participants as a marker for intestinal mucosa damage (Isnard *et al.*, 2020b). In agreement with the literature, we found a significant elevation of REG3α in the serum of male and female ART-HIV$^+$ donors, while this molecule was detected at very low levels in sera from HIV^{free} donors (supplemental Fig. 4B). Moreover, we found a significant correlation between the frequency of IRF-5 expression and the amount of REG3α in the serum of ART-HIV$^+$ donors (supplemental Fig. 4C), implying that DAMPs released from the damaged intestinal mucosa could trigger TLR7 and induce IRF-5.

In agreement with our findings in mice (Fabié *et al.*, 2018), IMQ treatment induced apoptosis (Zombie Aqua$^-$ AnnexinV$^+$ Caspase 3$^+$) (Fig. 3C) and cell death (Zombie Aqua$^+$ AnnexinV$^+$) (Fig. 3D) in memory CD4$^+$ T cells from ART HIV-1$^+$ patients to a significantly higher degree than in memory CD4$^+$ T cells from EC, directly correlating with TLR7 expression levels. Of note, IMQ treatment did not affect cell survival of CD4$^+$ T cells from HIV^{free} individuals (Fig.3D). Our results suggest that the TLR7-IRF-5 axis could play a role in memory CD4$^+$ T cell death in ART HIV-1$^+$ donors. This hypothesis was supported by the direct correlation between TLR7 and IRF-5 expression (Fig. 3E) and AnnexinV and TLR7 expression (Fig.3F). Although TLR7 and IRF5 are

also expressed in CD4⁺ T cells from ECs, no correlation between the expression of IRF-5 and TLR7, and TLR7 and AnnexinV was found in these cells (supplemental Fig. 5A and B).

Moreover, CD4⁺ T cell stimulation via TCR has been reported to induce IRF-5 in mice (Yan *et al.*, 2020). We found that the stimulation of cells with αCD3/αCD28 also induces IRF-5 in total (supplemental Fig. 5D) and memory CD4⁺ T cells (supplemental Fig. 5E) from ART-HIV-1⁺ and HIV^{free} individuals. However, this did not promote apoptosis (supplemental Fig. 5F) or cell death (supplemental Fig. 5G).

Figure 3.3

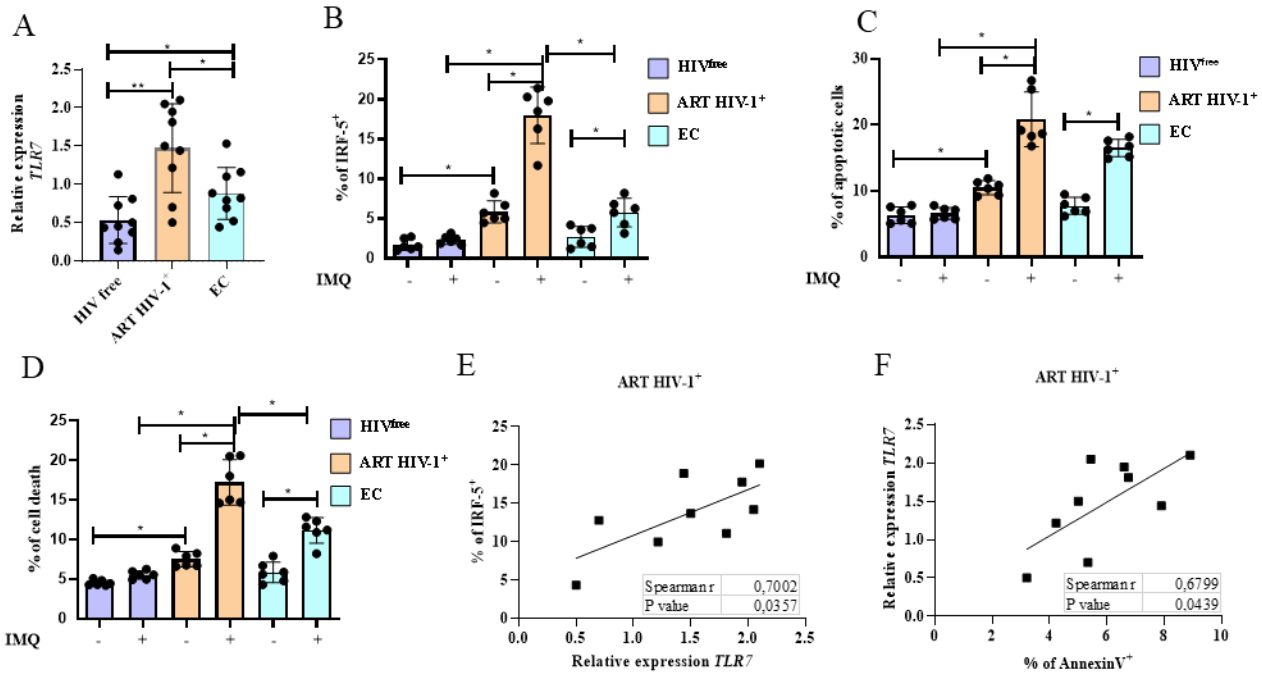


Figure 3.3 TLR7 is upstream of IRF-5

A) Graph shows *TLR7* mRNA levels in CD4⁺ T cells from ART HIV-1⁺, EC, and HIV^{free} individuals analyzed ex-vivo by RT- PCR. The Mann Whitney test was used to determine significance, * p <0.05, ** p< 0.01, n=9. CD4⁺ T cells purified from ART HIV-1⁺, EC, and HIV^{free} donors were treated *in vitro* with imiquimod (IMQ) or medium alone for 30h. Graphs represent B) the percentage of IRF-5⁺, C) the percentage of apoptotic, and D) the percentage dead memory CD4⁺ T cells. Data are presented as the mean ± SD. The Wilcoxon test was used to determine statistical differences, * p <0.05, n=6. Graphs show correlations between E) *TLR7* and IRF-5 expression and F) Annexin V and TLR7 expression in ART HIV-1⁺ donors. The Spearman r test was used to determine statistical significance, * p <0.05, n=9.

3.4.4 Caspase 8 is downstream of the TLR7-IRF-5 pathway

Several molecules involved in cell death have been described to be downstream molecular targets of IRF-5, including Fas, DR5, and Caspase 8, among others (Hu *et al.*, 2009). As it was previously reported that memory CD4$^+$ T cell death during HIV-1 infection is Fas-mediated (van Grevenynghe *et al.*, 2008b), and IRF-5 is required for Fas-induced apoptosis (Couzinet *et al.*, 2008), we set out to investigate whether Fas was downstream of IRF-5 in human CD4$^+$ T cells. In agreement with the literature (van Grevenynghe *et al.*, 2008b), we found no difference in the expression of Fas between any of the groups studied (Fig. 4A). Hence, we investigated whether Caspase 8 expression differed between the various groups of study. A significant upregulation of *CASP8* mRNA levels was observed in CD4$^+$ T cells from ART HIV-1$^+$ donors when compared with EC and HIV^{free} individuals (Fig. 4B). Moreover, when we treated CD4$^+$ T cells with IMQ, memory CD4$^+$ T cells from ART HIV-1$^+$ but not HIV^{free} individuals upregulated *CASP8* mRNA expression (Fig. 4C). Taken together, our results suggest that the TLR7-IRF-5 axis induces Caspase 8 expression and appears to be active in memory CD4$^+$ T cells from ART HIV-1$^+$ donors.

Figure 3.4

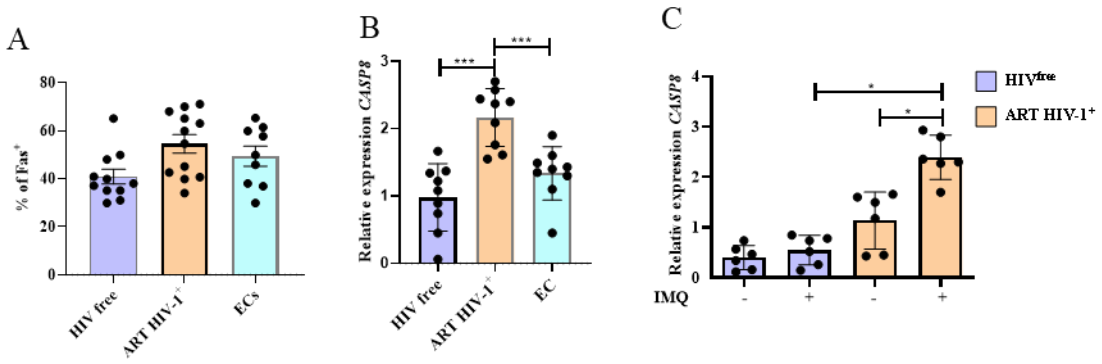


Figure 3.4 Caspase 8 is downstream of IRF5

CD4$^+$ T cells from PBMCs of ART HIV-1$^+$, EC, and HIV^{free} individuals were analyzed ex-vivo. Graphs show A) the percentage of Fas$^+$ memory CD4$^+$ T cells determined by flow cytometry, and B) RT-PCR analysis of *CASP8* mRNA levels in purified CD4$^+$ T cells. Data are presented as the mean ± SD. The Mann-Whitney *U*-test was used to determine statistical differences, * p <0.05, n=12 (ART HIV-1$^+$ and HIV^{free}), n=9 (EC). C) Purified CD4$^+$ T cells from ART HIV-1$^+$ and HIV^{free} donors were treated *in vitro* with imiquimod (IMQ) or medium alone for 30h. Graph show expression of *CASP8* mRNA. Data are presented as the mean ± SD. The Wilcoxon test was used to determine statistical significance, * p <0.05, n=6.

3.4.5 Activation of the TLR7/IRF-5 axis predisposes cells to Fas-mediated apoptosis

IRF-5 has been shown to be required for Fas-mediated cell death (Couzinet *et al.*, 2008). So, we investigated whether Fas/FasL interaction could also induce IRF-5. When we treated $CD4^+$ T cells with recombinant human Fas ligand (rFasL), we observed higher percentages of IRF-5^+ memory $CD4^+$ T cells in both ART HIV-1^+ and HIV^{free} individuals (Fig. 5A). As IRF-5 and Caspase 8 are also part of the Fas/FasL signalling pathway (Couzinet *et al.*, 2008; Hitoshi *et al.*, 1998; Zhang *et al.*, 1998), we hypothesized that activation of the TLR7-IRF-5-Caspase 8 axis would predispose cells to Fas-mediated apoptosis and enhance cell death. To prove our hypothesis, we pre-treated cells with or without IMQ for 18h, followed by a 12h incubation with rFasL, and finally analyzed the frequency of apoptotic and dead memory $CD4^+$ T cells (Fig. 5B). As expected, we detected significantly higher frequencies of apoptotic (Fig. 5C) and dead cells (Fig. 5D) in $CD4^+$ T cells from ART HIV-1^+ compared with HIV^{free} individuals following incubation with rFasL alone, suggesting that ART HIV-1^+ $CD4^+$ T cells had an intrinsic higher predisposition to die by Fas-mediated apoptosis than HIV^{free} $CD4^+$ T cells. We also noticed a synergistic effect in the induction of apoptotic cells (Fig. 5C) and cell death (Fig.5D) when we treated purified $CD4^+$ T cells with IMQ followed by rFasL, when compared with cells incubated with rFasL alone. This effect was less pronounced in $CD4^+$ T cells from HIV^{free} than from ART HIV-1^+ individuals. We next analyzed *CASP8* mRNA levels by RTqPCR and found a synergism of the stimulation with IMQ followed by rFasL in the the induction of *CASP8* mRNA in $CD4^+$ T cells from ART HIV-1^+ donors only, when compared with rFasL alone (Fig. 5E). Taken together, our results show that the TLR7-IRF-5 pathway feeds into the Fas/FasL signalling pathway, which subsequently amplifies IRF-5 and Caspase 8 expression, creating a spiral of death.

Figure 3.5

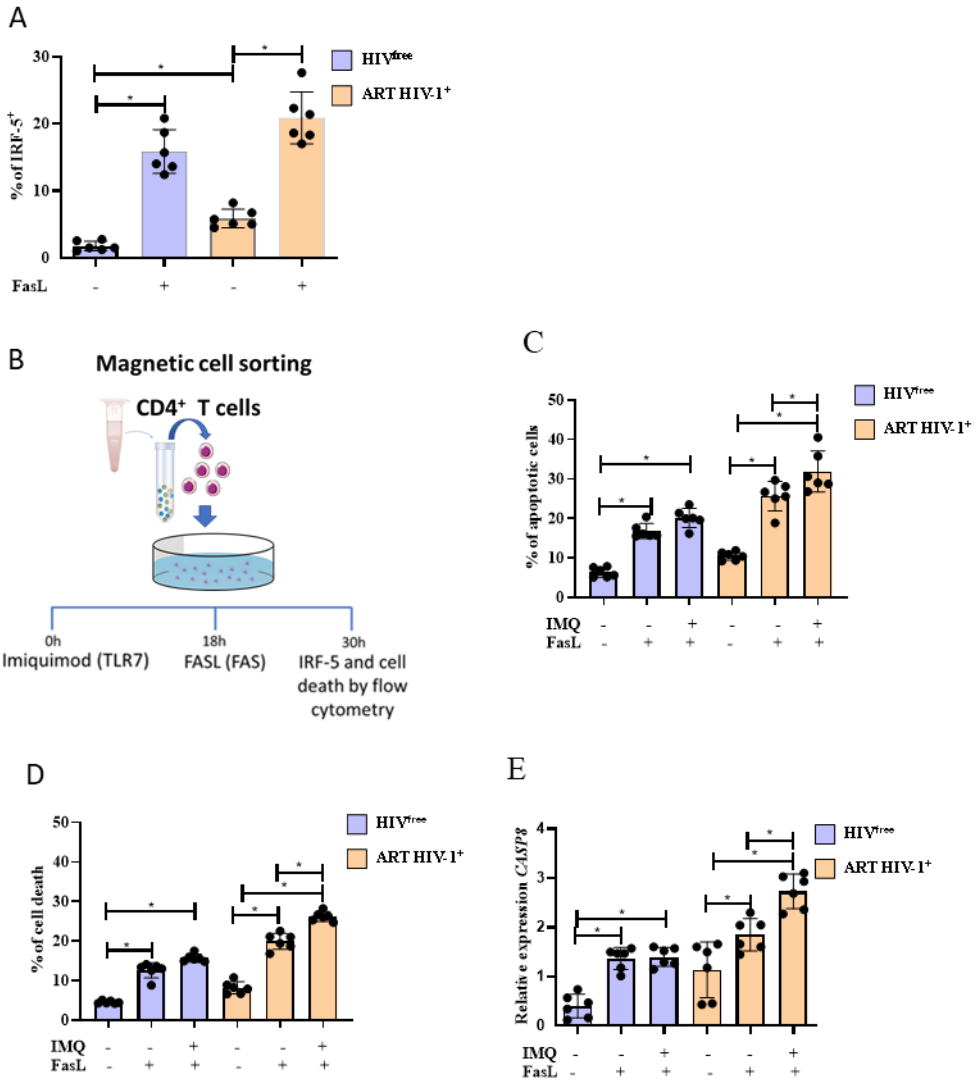


Figure 3.5 Activation of the TLR7-IRF-5 axis predisposes memory CD4⁺ T cells to Fas-mediated apoptosis

A) Purified CD4⁺ T cells from ART HIV-1⁺ and HIV^{free} donors were incubated *in vitro* with rFasL or medium alone for 18h. Graph shows IRF-5 expression as measured by flow cytometry. Data are presented as the mean ± SD. The Wilcoxon test was used to determine statistical differences, * p <0.05, n=6. B) Scheme of the experimental design used for 5C-E: purified CD4⁺ T cells were treated with IMQ or medium alone; 12h later rFasL or medium were added to the culture and the cells were incubated for further 18h for a total of 30h incubation at 37°C. Graphs show C) the percentage of apoptotic memory CD4⁺ T cells, D) the percentage of dead memory CD4⁺ T cells, and E) *CASP8* mRNA levels in memory CD4⁺ T cells after 30h stimulation. Data are presented as the mean ± SD. The Wilcoxon test was used to determine statistical significance, * p <0.05, n=6.

3.4.6 IRF-5 inhibitory peptides limit IMQ and Fas/FasL induced cell death

Our findings so far indicate that IRF-5 is a key molecule in both the TLR7-IRF-5 axis and the Fas/FasL signalling pathway. Hence, we finally sought to block IRF-5 activation to limit cell death. To this end, we decided to use IRF-5 inhibitory peptides developed by Banga et al. (Banga *et al.*, 2020). These cell permeable peptides (IRF-5-CPPs) efficiently block IRF-5 function downstream of IRF-5 phosphorylation, preventing subsequent IRF-5 activation in various cell types (Banga *et al.*, 2020); however, IRF-5-CPPs have yet to be tested in T cells. Thus, we chose four different peptides (P1-4) with different cell-specificities (see Table 3.2). We first tested the cytotoxicity of different IRF-5-CPPs, including the control peptide, on human $CD4^+$ T cells from HIV^{free} donors at concentrations varying from 0 to 50µM. IRF-5-CPPs were found to be non-cytotoxic up to 20µM (supplemental Fig. 6A). When we tested the ability of IRF-5-CPPs to inhibit nuclear localization of human IRF-5 in untreated and IMQ-stimulated $CD4^+$ T cells from ART HIV-1^+ and HIV^{free} donors, we observed that P1 and P3 were the most effective peptides at preventing IRF-5 activation in unstimulated $CD4^+$ T cells (supplemental Fig. 6B; about 62% and 60% inhibition following P1 and P3 treatment, respectively) as well as in IMQ-treated (supplemental Fig. 6C) cells. We also tested whether P1 and P3 could be used together to achieve a stronger IRF-5 blockade and ultimately inhibit IMQ-induced cell death. To this end, $CD4^+$ T cells from ART HIV-1^+ and HIV^{free} individuals were treated with either 5µM P1 or P3 alone, or with a mixture of P1 and P3, and subsequently stimulated with IMQ. As shown in supplemental Figure 3.15D, the P1/P3 mix was slightly more efficient in blocking IRF-5 activation in ART HIV-1^+ $CD4^+$ T cells upon stimulation with IMQ. Indeed, we observed 44% inhibition of IRF-5 nuclear localization following treatment with P1, 40% after incubation with P3, and about 60% when cells were co-cultured with P1 and P3 together. Thus, we decided to use a combination of P1 and P3 to inhibit the TLR7-IRF-5 pathway; the control peptide was used for all "$Peptide^{neg}$ control groups. To this end, we treated purified $CD4^+$ T cells with the peptide mix for 30 minutes before stimulation with IMQ. The combination of the two IRF-5-CPPs was able to significantly block apoptosis in memory $CD4^+$ T from ART HIV-1^+ patients. Indeed, we observed lower frequencies of apoptotic (Fig. 6A) and dead (Fig. 6B) cells in memory $CD4^+$ T from ART HIV-1^+ patients following IMQ stimulation. As expected, this was not the case in HIV^{free} donors, as these cells express very low levels of TLR7 and do not respond to IMQ (Fig. 3A, C, and D). Similar results were obtained when we analysed *CASP8* mRNA levels, which were significantly reduced when we added IRF-5 blocking peptides to purified $CD4^+$ T cells from ART HIV-1^+ patients stimulated with IMQ (Fig. 6C).

As the Fas/FasL pathway is involved in memory $CD4^+$ T cell death in HIV-1 infection (van Grevenynghe *et al.*, 2008b), we next assessed whether the IRF-5-CPP mix could block Fas-mediated apoptosis in memory $CD4^+$ T cells from ART HIV-1^+ patients. $CD4^+$ T cells were incubated for 1 hour with 5 µM of a mixture of P1 and P3 or the control peptide, followed by an 18h-incubation with or without IMQ. Cells were then treated with rFasL for another 12h (Fig. 6D). Remarkably, the IRF-5-CPP mix could significantly limit Fas-mediated apoptosis. In fact, the increase in the frequency of apoptotic cells (Fig. 6E) or dead cells (Fig. 6F) following rFasL or IMQ + rFasL treatment was approximately reduced by half after IRF-5 blockade in $CD4^+$ T cells from ART HIV-1^+ donors. A similar effect but less pronounced effect was observed in $CD4^+$ T cells from HIV^{free} individuals. No differences were observed between $CD4^+$ T cells from male and female donors, independently of whether they were HIV^+ or HIV^{free} (supplemental Fig. 7 A-D).

Finally, to clearly prove that inhibition of Fas-mediated apoptosis was indeed dependent on IRF-5 blockade, we assessed *CASP8* mRNA levels after the various treatments, Caspase 8 being a downstream molecular target of IRF-5 (Hu *et al.*, 2009). We observed a nearly 50% reduction in the induction of *CASP8* mRNA in $CD4^+$ T cells from ART HIV-1^+ donors treated with either rFasL or IMQ + rFasL following IRF-5 blockade (Fig. 6G). The effect in memory $CD4^+$ T cells from HIV^{free} samples was only partial and less pronounced than observed in ART HIV-1^+ $CD4^+$ T cells (Fig. 6G). We did not notice any difference between $CD4^+$ T cells from male and female donors (supplemental Fig. 7 E). Taken together, our results suggest that IRF-5 plays a central role in ART HIV-1^+ memory $CD4^+$ T cell death, not only as a downstream target of TLR7 but also by being directly involved in Fas-mediated apoptosis.

Figure 3.6

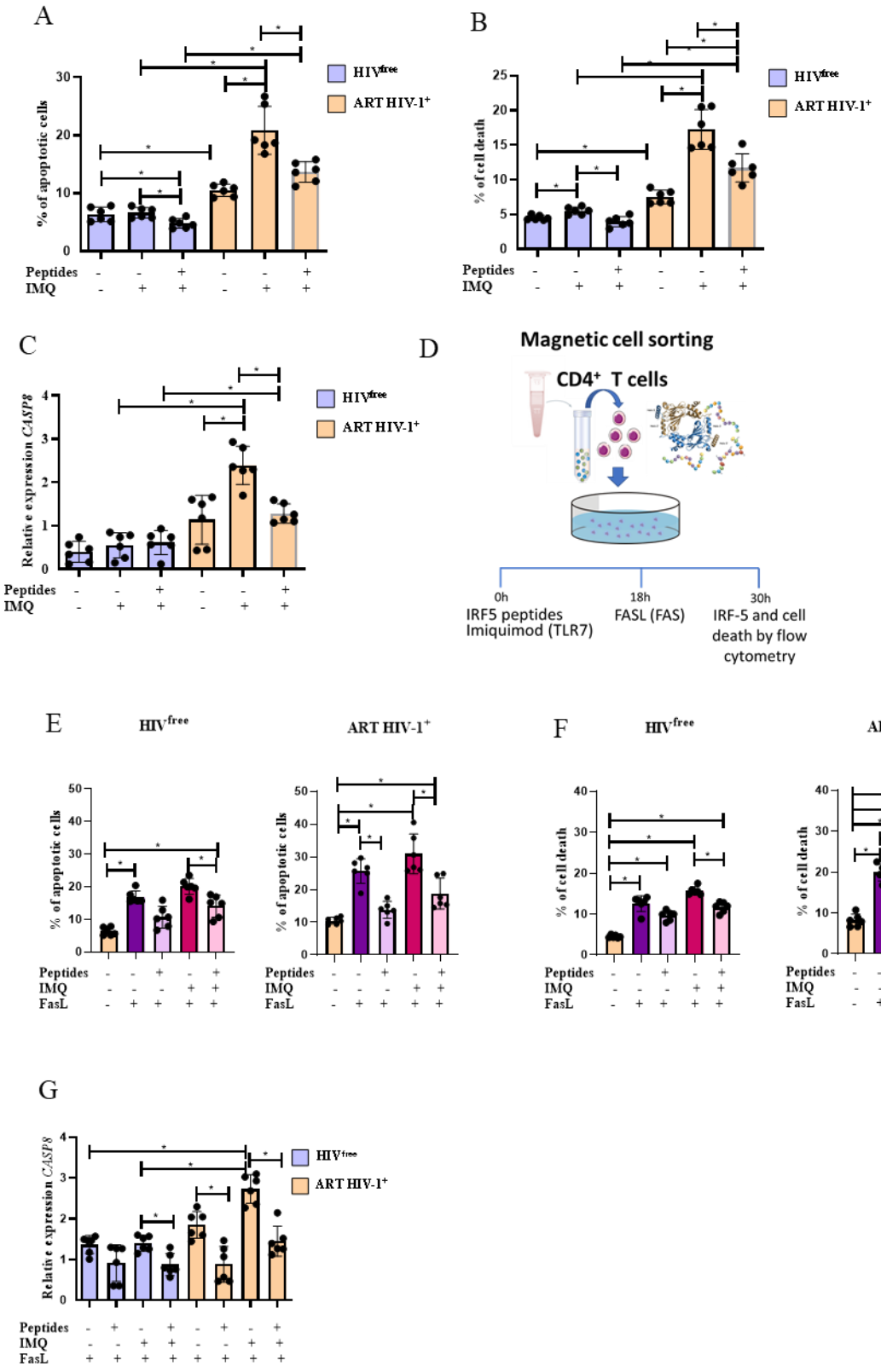

A
% of apoptotic cells
HIV free
ART HIV-1+
Peptides
IMQ
B
% of cell death
C
Relative expression CASP8
D
Magnetic cell sorting
CD4+ T cells
0h
IRF5 peptides
Imiquimod (TLR7)
18h
FASL (FAS)
30h
IRF-5 and cell death by flow cytometry
E
F
FasL
G

Figure 3.6 IRF-5 inhibitory peptides rescue memory CD4⁺ T cells from Fas-mediated apoptosis

A-C) Purified CD4⁺ T cells from ART HIV-1⁺ and HIV^{free} individuals were pretreated with 10 μM of IRF-5-CPPs for 30 min before stimulation with IMQ and incubated at 37°C for 30h. Graphs show A) the percentage of apoptotic cells, B) the percentage of dead cells, and C) *CASP8* mRNA expression levels in memory CD4⁺ T cells from ART HIV-1⁺ and HIV^{free} donors. D) Experimental design: Purified CD4⁺ T cells from ART HIV-1⁺ and HIV^{free} individuals were pretreated with 10 μM of IRF-5-CPPs for 30 min, stimulated with or without IMQ for 12h and incubated for further 18h with or without rFasL. Graphs show E) the percentage of apoptotic cells, F) the percentage of dead cells, and G) *CASP8* mRNA expression levels in memory CD4⁺ T cells from ART HIV-1⁺ and HIV^{free} donors. Data are presented as the mean ± SD. The Wilcoxon test was used to determine statistical significance, * $p < 0.05$, n=6.

3.4.7 IFN-β and DAMPs promote TLR7 expression in CD4⁺ T cells

Although TLR7 expression on T cells has been reported in mice and humans (Caramalho *et al.*, 2003; Dominguez-Villar *et al.*, 2015; Gelman *et al.*, 2004; Song *et al.*, 2009), the signaling pathways promoting TLR7 upregulation in CD4⁺ T cells remain unknown. Type I IFN was shown to enhance TLRs' expression in B cells (Doucett *et al.*, 2005; Green *et al.*, 2009b; Thibault *et al.*, 2009) (Silva-Barrios *et al.*, 2016a). As IFN-ß is often part of chronic inflammatory environments associated with persistent infections (Dillon *et al.*, 2018) and TLR7 is upregulated in CD4⁺ T cells during persistent infections (Fabié *et al.*, 2018), we first assessed whether purified CD4⁺ T cells from HIV^{free} individuals exposed to recombinant IFN-ß would upregulate TLR7. We found that IFN-ß promoted *TLR7* mRNA upregulation as early as 6 hours after initial exposure and this expression was maintained over 24 hours (Fig. 7A). Tissue disruption and release of DAMPs are also hallmarks of chronic inflammatory environments. Thus, we next exposed CD4⁺ T cells from HIV^{free} donors to apoptotic cell material. Interestingly, apoptotic cell material could also enhance *TLR7* mRNA expression, although to a lesser extent than IFNß; nevertheless, its effect was synergistic with IFN-ß (Fig. 7B). This suggests that CD4⁺ T cells in an inflammatory environment can upregulate TLR7 independently from their antigen-specificity. Following this, we assessed whether CD4⁺ T cell priming in the presence of DAMPs and IFN-ß can modulate *TLR7* mRNA expression levels. Stimulation with anti-CD3/CD28 in the presence or absence of apoptotic cell material slightly induced *TLR7* expression (Fig. 7B). However, anti-CD3/CD28 stimulation of cells previously exposed to IFN-ß greatly promoted *TLR7* expression, which was further enhanced in the presence of DAMPs (Fig. 7B), indicating that antigen specific CD4⁺ T cells primed in a persistent inflammatory environment rich in IFN-ß and DAMPs are bound to express high levels of *TLR7*.

To test whether $CD4^+$ T cells exposed to IFN-ß and/ or apoptotic cell material with or without anti-CD3/CD28 stimulation were more susceptible to Fas-mediated apoptosis, we treated cells with rFasL. When we analysed memory $CD4^+$ T cells, we found higher frequencies of IRF-5^+ cells (Fig. 7C), apoptotic cells (Fig. 7D), and dead cells (Fig. 7E) when $CD4^+$ T cells were exposed to IFNß + DAMPs prior to rFasL treatment, compared with rFasL alone. Anti-CD3/CD28 stimulation did not alter the cells' susceptibility to Fas-mediated apoptosis (Fig. 7G and H) but slightly increased IRF-5 expression (Fig. 7F). In agreement with the *TLR7* expression levels shown in Figure 7B, $CD4^+$ T cells exposed to IFNß + DAMPs prior to anti-CD3/CD28 stimulation were significantly more susceptible to Fas-mediated apoptosis (Fig. 7G and H) and expressed the highest levels of IRF-5 (Fig. 7F) of any treatment group. Similar results were obtained when we analyzed memory $CD4^+$ T cells (supplemental Fig. 8 A-F). Taken together, these results suggest that exposure of antigen-specific or bystander $CD4^+$ T cells to apoptotic material and IFNß promotes the upregulation of TLR7 and predisposes cells to FAS-mediated cell death.

Figure 3.7

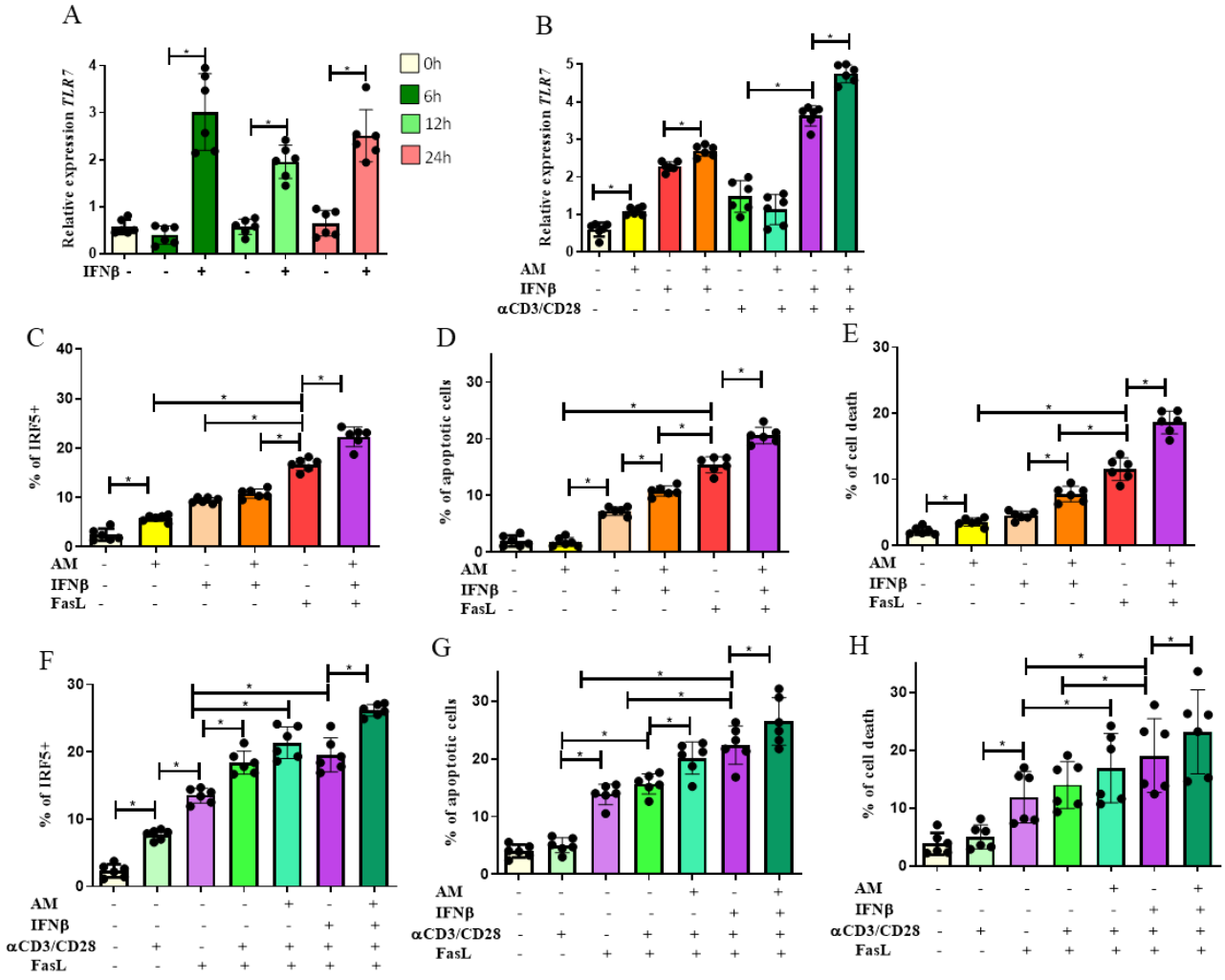


Figure 3.7 IFNβ and DAMPs promote the upregulation of TLR7 on CD4⁺ T cells

A) Purified CD4⁺ T cells from HIV^{free} individuals were incubated with IFNβ or with medium alone. Graph shows RT PCR analysis of *TLR7* expression at 0, 6, 12 and 24h of treatment. B) Purified CD4⁺ T cells from HIV^{free} individuals were treated with IFNβ in the presence or absence of conditioned medium with 10% v/v apoptotic material (AM, supernatant of staurosporine-treated PBMCs) for 24 h, before stimulation with αCD3α/CD28 for further 24h. Graphs shows RT PCR analysis of *TLR7* gene expression. C-E) Purified CD4⁺ T cells from HIV^{free} individuals were incubated with IFNβ in the presence or absence of conditioned medium with 10% v/v apoptotic material for 24 h, before adding rFasL for further 18h. Graphs represent C) the percentage of IRF-5⁺, D) the percentage of apoptotic, and E) the percentage of dead CD4⁺ T cells after incubation with the indicated culture conditions. F-H) Purified CD4⁺ T cells from HIV^{free} donors were treated with IFNβ in the presence or absence of conditioned medium with 10% v/v apoptotic material for 24 h; they were then stimulated with αCD3/αCD28 for further 24h and finally incubated for 18h with rFasL. Graphs represent F) the percentage of IRF5⁺, G) the percentage of apoptotic, and H) the percentage of dead CD4⁺ T cells after incubation

as described above. Data are presented as the mean ± SD. The Wilcoxon test was used to determine statistical significance, * $p < 0.05$, n=6.

In summary, we propose that memory $CD4^+$ T cells from ART HIV-1^+ patients express TLR7 and IRF-5, which represent an imprint that predisposes these cells to Fas-mediate apoptosis. The TLR7-IRF-5 axis can be activated by DAMPs, resulting in heightened Caspase 8 expression. This pathway directly feeds into the Fas-FasL pathway, which increases IRF-5 and Caspase 8 expression leading to cell death. In contrast, memory $CD4^+$ T cells from HIV^{free} individuals and from ECs, express low levels of TLR7 and IRF-5, which makes these cells less capable of sensing DAMPs and ultimately less prone to Fas-mediated apoptosis (Figure 3.8). Figure 3.9 summarizes how this pathway could work synergistically with other known cell death pathways (reviewed by refs.(Cummins *et al.*, 2010a; Doitsh *et al.*, 2016)), particularly those requiring Caspase 8, to induce apoptosis in CD4+ T cells during PHI and after ART.

Figure 3.8

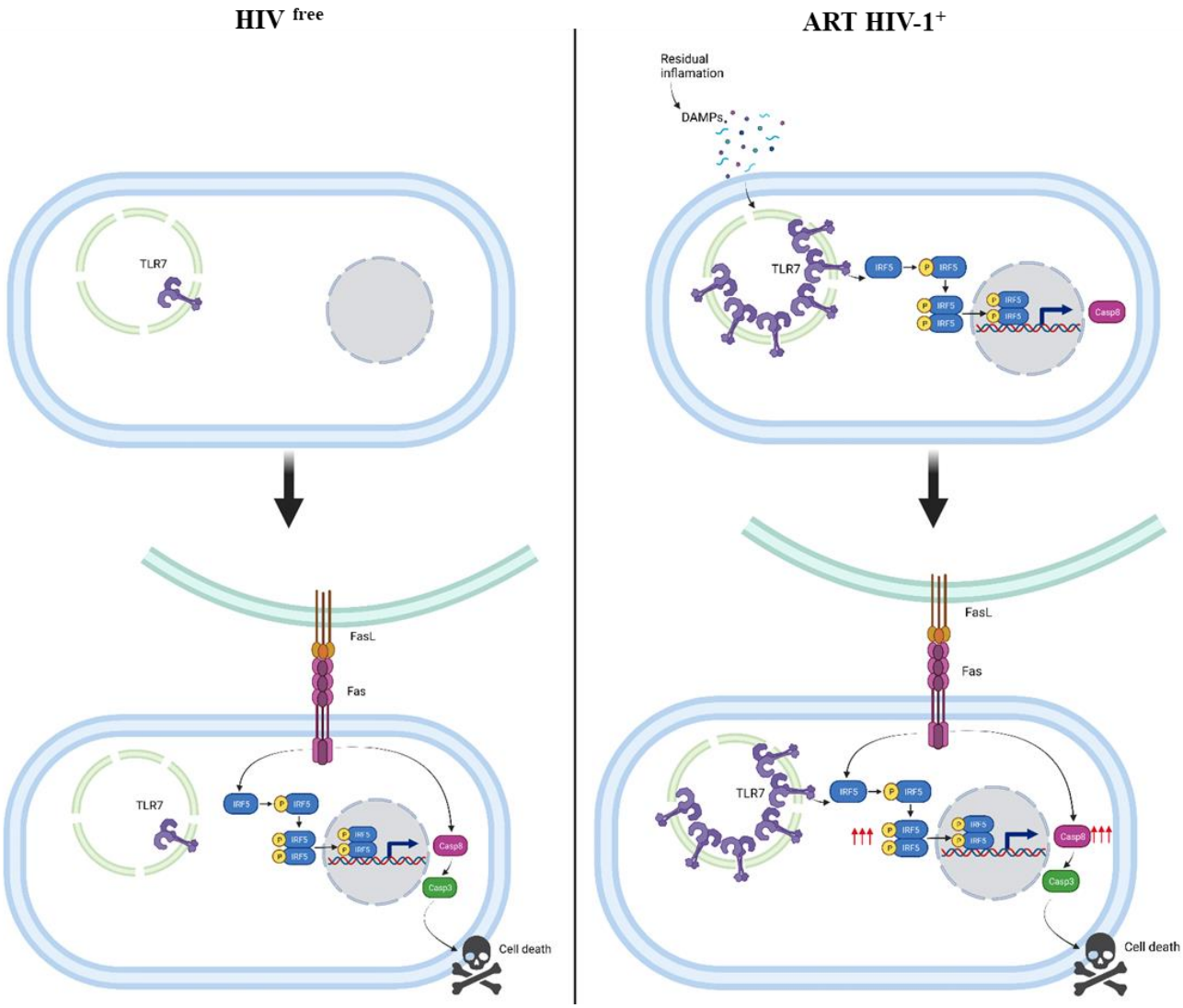
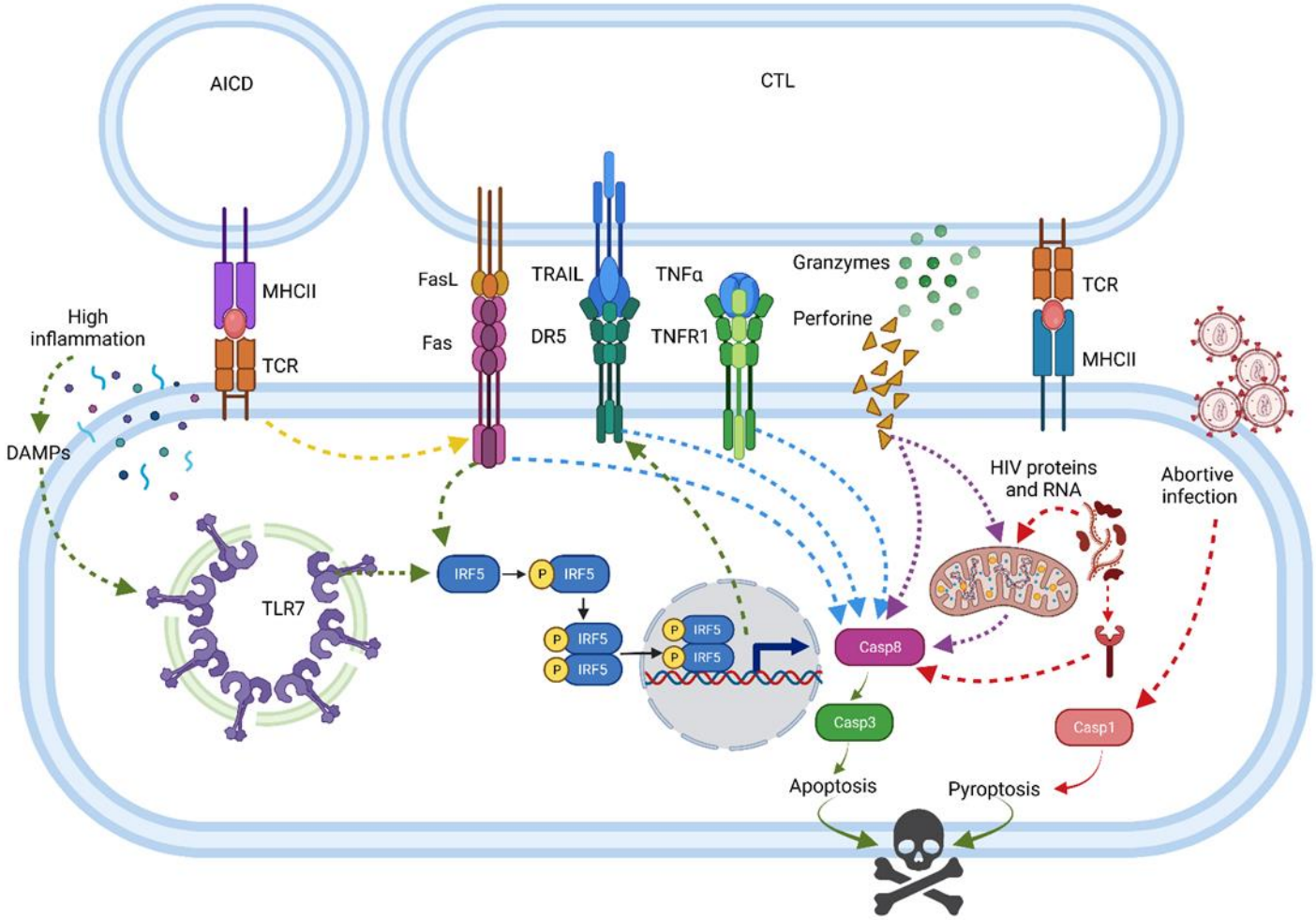


Figure 3.8 Proposed model

Residual inflammation maintains the upregulation of TLR7 and IRF-5 in memory CD4 $^{+}$ T cells from ART HIV-1^{+} individuals, but not in HIV^{free}. The activation of this TLR7-IRF-5 axis feeds into the Fas/FasL pathway predisposing and enhancing Fas-mediated apoptosis only in memory CD4 $^{+}$ T cells from ART HIV-1^{+} individuals. Finally, we proposed that this mechanism can be block by adding IRF-5 inhibitory peptides, which block the predisposition to Fas-mediated cell death. Created with BioRender.com.

Figure 3.9

PHI



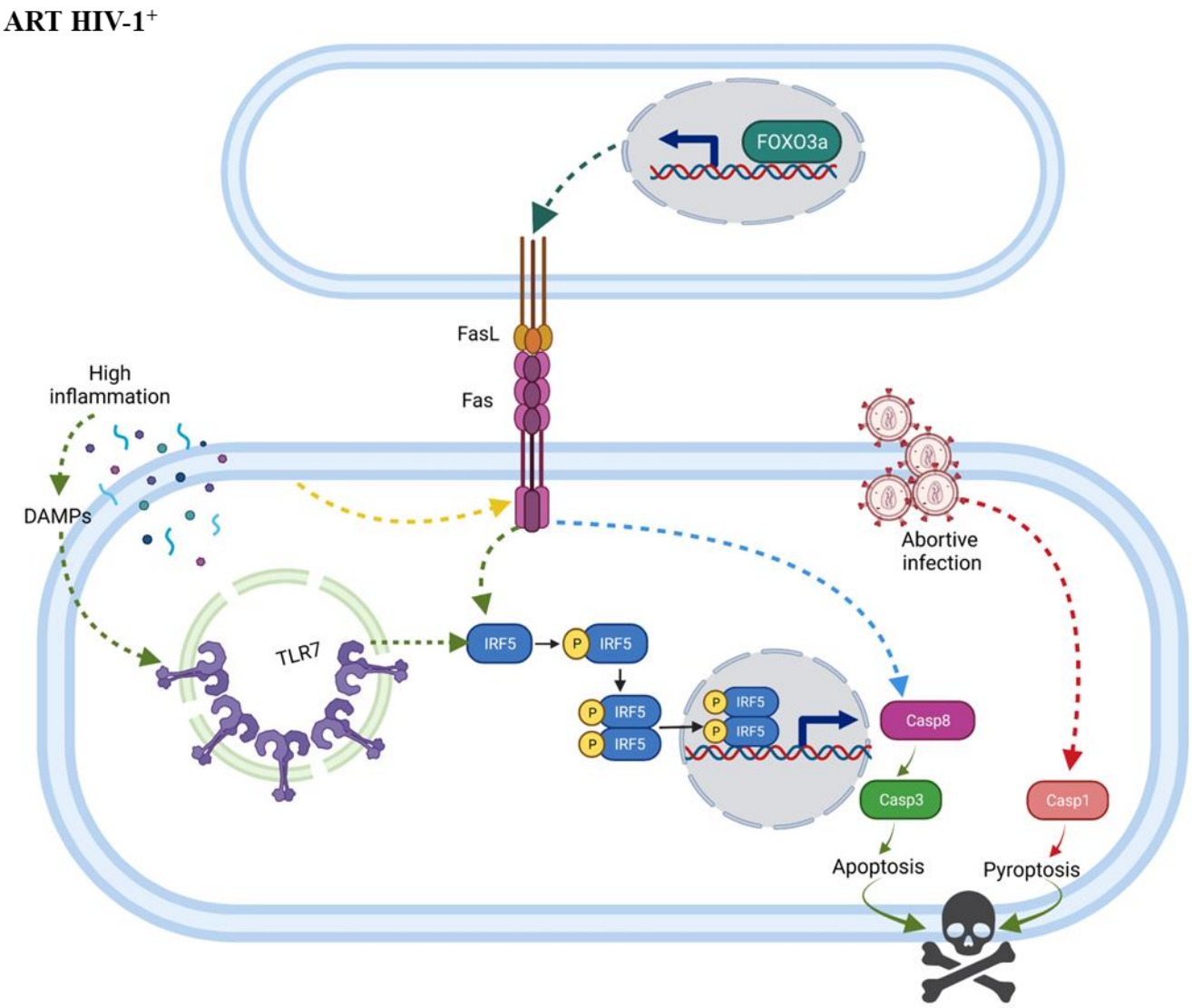


Figure 3.9 Summary of cell death pathways known to occur in CD4⁺ T cells during primary HIV-1 infection or following ART. PHI

A large proportion of CD4⁺ T cell death during primary HIV-1 infection occurs via apoptosis and comprises extrinsic and intrinsic pathways. Extrinsic apoptosis involves death receptor members of the tumor necrosis factor receptor gene superfamily, such as Fas, TNFR1, DR5 (TRAILR2). The activation of these receptors by a death ligand results in the activation of caspase 8. We propose that the TLR7-IRF-5 pathway feeds into these pathways by sensitizing cells to Fas-, TNFR1-, and DR5-mediated apoptosis through the induction of Caspase 8 and IRF-5. Other extrinsic cell death pathways include activation-induced cell death (AICD) and killing by CTLs via secretion of perforin and granzymes. Intrinsic apoptosis includes activation of Caspase 3 by HIV proteins and DNA.

Finally, abortively infected CD4⁺ T cells die by pyroptosis, a highly inflammatory form of programmed cell death that results from the activation of Caspase 1. **ART HIV-1⁺**: Although antiretroviral therapy restores CD4+ T cell counts, memory CD4⁺ T cells from ART HIV-1⁺ patients are still prone to apoptosis, despite treatment. These cells mostly die

by Fas-mediated apoptosis. The Fas/FasL pathway is strengthen on one hand by the transcription factor Foxo3a, which empowers the “killers”, and, on the other hand, by the TLR7-IRF-5 axis, which weakens the “victims”. Death by pyroptosis in abortively infected cells may also occur. Created with BioRender.com.

3.5 Discussion

Although most HIV-1$^+$ patients recover CD4$^+$ T cell counts on ART it remains unclear why memory CD4$^+$ T cells are highly sensitive to apoptosis. Here we show that memory CD4$^+$ T cells from HIV$^+$ patients undergoing ART display an imprint by expressing higher levels of *TLR7* mRNA. We propose that this allows memory CD4$^+$ T cells to sense inflammatory tissue damage-derived DAMPs and activates the TLR7-IRF-5 pathway, making cells more susceptible to Fas-mediated apoptosis. Treatment with IRF-5 blocking peptides successfully blocks the TLR7-IRF-5 pathway and significantly reduces memory cell death by Fas-mediated apoptosis (Figure 3.8).

PLWH on ART are characterized by inflammation and express on CD4$^+$ T cells higher levels immune checkpoint molecules like PD-1, CTLA-4, LAG-3, and TIGIT compared with HIV^{free} participants (Fromentin *et al.*, 2016). In addition to their exhaustion function, these molecules contribute to the establishment and maintenance of a latent cellular infection (Banga *et al.*, 2016; Chomont *et al.*, 2009; Fromentin *et al.*, 2016; Rasmussen *et al.*, 2022). Our findings show that memory CD4$^+$ T cells from patients on ART also differ from those from HIV^{free} individuals and EC in their TLR7 expression. Following TLR7 triggering, IRF-5 is activated, which in turn promotes Caspase 8 in memory CD4$^+$ T cells from ART HIV-1$^+$ participants. A significant proportion of memory and effector CD4$^+$ T cells expressed active IRF-5 (localized to the nucleus) and upregulated *CASP8*, suggesting that the TLR7-IRF-5 axis is active in some cells. Still, the factors that activate TLR7 in CD4$^+$ T cells in ART treated PLWH remain to be unvealed.

In our previous work, we have shown that CD4$^+$ T cells are capable of sensing inflammatory tissue damage-derived DAMPs via TLR7 in a murine model of visceral leishmaniasis (Fabié *et al.*, 2018). As patients on ART display residual levels of immune activation and inflammation, it is possible that inflammation could be involved in generating DAMPs-derived from tissue damage occurring at the intestinal mucosal site (Brenchley *et al.*, 2006; Jenabian *et al.*, 2015), a major HIV reservoir. The fact that IRF-5 is highly expressed and active in CD4$^+$ T cells from PHI suggests that the level of inflammation, and consequently DAMPs release, is proportional to IRF-5 upregulation.

Nevertheless, activation of the TLR7-IRF-5 axis in $CD4^+$ T cells may be multifactorial and could also be triggered by microbial products released into the circulation from the gut lumen due to compromised gastrointestinal mucosa (Brenchley *et al.*, 2006). An additional source of DAMPs could be derived from material released by non-permissive, resting $CD4^+$ T cells dying by pyroptosis following abortive HIV infection (Doitsh *et al.*, 2014; Monroe *et al.*, 2014). Moreover, HIV-1 is an enveloped retrovirus with two copies of an ssRNA genome that may be recognized by TLR7 (Luban, 2012; Meas *et al.*, 2020). To this end, it would be interesting to analyse IRF-5 expression in $CD4^+$ T cells located at anatomical reservoir sites, like in the gut mucosa.

Independently from the agonist molecule that triggers TLR7, ART-treated PLWH are characterized by an imprint on $CD4^+$ T cells, likely occurring before ART, that makes them more susceptible to sensing DAMPs, HIV, or both. This imprint is also responsible for the activation of IRF-5 and consequently Caspase 8 and for sensitizing memory cells to Fas-mediated apoptosis. Interestingly, we show that TLR7 and IRF-5 expression levels directly correlate with $CD4^+$ T cell death in PLWH on ART. IRF-5 expression is also inversely correlated with $CD4^+$ T cell counts in PHI, suggesting that this pathway may also operate early during acute infection and could lead to the death of bystander $CD4^+$ T cells. Hence, activation of the TLR7-IRF-5 axis and subsequent predisposition to Fas-mediated apoptosis might also affect $CD4^+$ T cells' homeostasis in general and might not be solely restricted to the memory compartment. Thus, it would be interesting to investigate the role of IRF-5 in HIV-1^+ patients that do not respond to therapy, called immune non-responders (Autran *et al.*, 1997). Incomplete $CD4^+$ T cell recovery (below 350 cells, despite years on effective ART) has been associated with increased replicative senescence, increase in effector CD4, and higher sensitivity to cell death, all of which contribute to a lower proliferative capacity of $CD4^+$ T cells (Brenchley *et al.*, 2003; Fernandez *et al.*, 2006; Lederman *et al.*, 2011; Marchetti *et al.*, 2006; Massanella *et al.*, 2013). More importantly, the extent of lymphoid tissue damage has been shown to influence $CD4^+$ T cell count reconstitution (Zeng *et al.*, 2012), suggesting that the level of inflammation and the extent of inflammatory tissue damage at the time of therapy affects $CD4^+$ T cell recovery. Tissue disruption results in the release of DAMPs, which could trigger TLR7 expressed on $CD4^+$ T cells and activate the TLR7-IRF-5 axis. Hence, IRF-5 blockade could be beneficial in PLWH that fail to recover or show a slow recovery of $CD4^+$ T cells after ART.
Of note, despite having an active infection and higher levels of systemic immune activation compared to HIV^{free} individuals (Hunt *et al.*, 2008), $CD4^+$ T cells from EC donor expressed low levels of TLR7, insufficient to promote IRF-5 and cell death.

TLR7 agonists, in combination with other therapies, have been shown to promote sustained control of simian-human immunodeficiency virus (SHIV) or SIV in nonhuman primates (Borducchi *et al.*, 2018; Hsu *et al.*, 2021; Kasturi *et al.*, 2020; Lim *et al.*, 2018; Moldt *et al.*, 2022; Walker-Sperling *et al.*, 2022). Indeed, the TLR7 agonist GS-9620 was shown to activate HIV in PBMCs from ART HIV$^+$ patients and to reduce viral replication via the production of IFNα (Bam *et al.*, 2017; Tsai *et al.*, 2017). However, only modest effects were observed in HIV controllers on ART who received an oral TLR7 agonist (SenGupta *et al.*, 2021). The mild effect was to be attributed to increased dendritic cell and natural killer cell crosstalk and an increase in cytotoxicity potential (SenGupta *et al.*, 2021). Similarly to ECs, rhesus macaques are capable of naturally controlling SIV infection. It is thus possible that their CD4$^+$ T cells do not express high levels of TLR7. In this case, the use of TLR7 agonists will not affect the memory CD4$^+$ T cell compartment. However, our results suggest that the therapeutic use of TLR7 agonists could lead to an imbalance in CD4$^+$ T cell homeostasis and a loss of memory and effector CD4$^+$ T cells in HIV$^+$ patients on ART with CD4$^+$ T cells expressing high TLR7 levels.

Several important questions arose from our study: i) what induces TLR7? ii) what determines TLR7 expression levels in CD4$^+$ T cells? iii) Are TLR7 expression levels associated with the capacity to maintain CD4$^+$ T cell homeostasis during primary infection?

We tried to identify possible pathways that could lead to TLR7 upregulation in CD4$^+$ T cells and found that IFNβ together with DAMPs released from tissue damage display a synergistic effect in inducing TLR7 expression on CD4$^+$ T cells. Although this possible synergism can be enhanced by TCR triggering, TCR engagement is not strictly required. These results imply that the level of IFN-I response and inflammation may determine the level of TLR7 that CD4$^+$ T cells (antigen-specific and bystanders) will be imprinted with. It is largely recognized that TLR7 expression on innate immune cells, particularly plasmacytoid dendritic cells (pDCs), is essential for inducing the IFN-I response, which is required to control HIV spread during the acute phase of infection (Li *et al.*, 2014; Sandler *et al.*, 2014). However, IFN-I may also have immunosuppressive effects in HIV infection (Campillo-Gimenez *et al.*, 2010; Jacquelin *et al.*, 2009). Hence, a balance between the double-edge IFN-I functions, antiviral and immune suppression, is crucial for the outcome of HIV infection (Telenti, 2014). One reason for varying IFN-I levels in different individuals is via TLR7 polymorphisms (Azar *et al.*, 2020; Fallerini *et al.*, 2021; Shaikh *et al.*, 2019; Shi *et al.*, 2020; Zaidane *et al.*, 2020a; Zhang *et al.*, 2020), which could determine the amount of IFN-I that is produced (Azar *et al.*, 2020). Polymorphisms in the *IRF5* gene could also explain why the TLR7-

IRF-5 axis is more activated in certain individuals than others. IRF5 polymorphisms have been well described for autoimmune disease and are often associated with various levels of inflammation (Dideberg *et al.*, 2007; Dieguez-Gonzalez *et al.*, 2008; Graham *et al.*, 2006a; Hedl *et al.*, 2012).

Our findings highlight the central role played by IRF-5, which is not only involved in predisposing memory $CD4^+$ T cells to Fas-mediated apoptosis, but also a key component of the Fas/FasL pathway. The IRF-5 effects are complementary to Foxo3a, which enhances the expression of FasL (van Grevenynghe *et al.*, 2012; van Grevenynghe *et al.*, 2008b), empowering the “killers”, while IRF-5 promotes the expression of Fas (Hu *et al.*, 2009), weakening the “victims”. It is thus not surprising that IRF-5 blockade limited not only the induction of caspase 8 via the TLR7-IRF-5 axis but also Fas-mediated cell death. IRF-5 blockade could be developed as a possible therapy to prevent the loss of protective memory $CD4^+$ T cells in patients under ART and to help recover $CD4^+$ T cell counts in HIV^+ patients that do not respond to therapy. As a heightened expression of IFN-I and tissue damage are common features of persistent inflammatory responses, it is possible that the pathway we describe in this study is not only involved in HIV-1-pathogenesis but could also operate during other chronic viral infections, such as SARS-CoV2.

In conclusion, we report that $CD4^+$ T cells from PLWH receiving ART display heightened *TLR7*, IRF-5, and *CASP8* expressions compared to $CD4^+$ T cells from EC and HIV^{free} donors. Moreover, activation of the TLR7-IRF-5 axis feeds into the Fas/FasL pathway predisposing and enhancing Fas-mediated apoptosis. The transcription factor IRF-5 plays a central role in both pathways and blockade of IRF-5 activation limits cell death. We propose IRF-5 blockade as a possible therapeutic target to prevent memory $CD4^+$ T cell loss in patients under ART and, more importantly, for patients who have a low $CD4^+$ T cell recovery despite long-term ART that represent 20 % of patients with a persistent risk to develop non-AIDS and AIDS comorbidities.

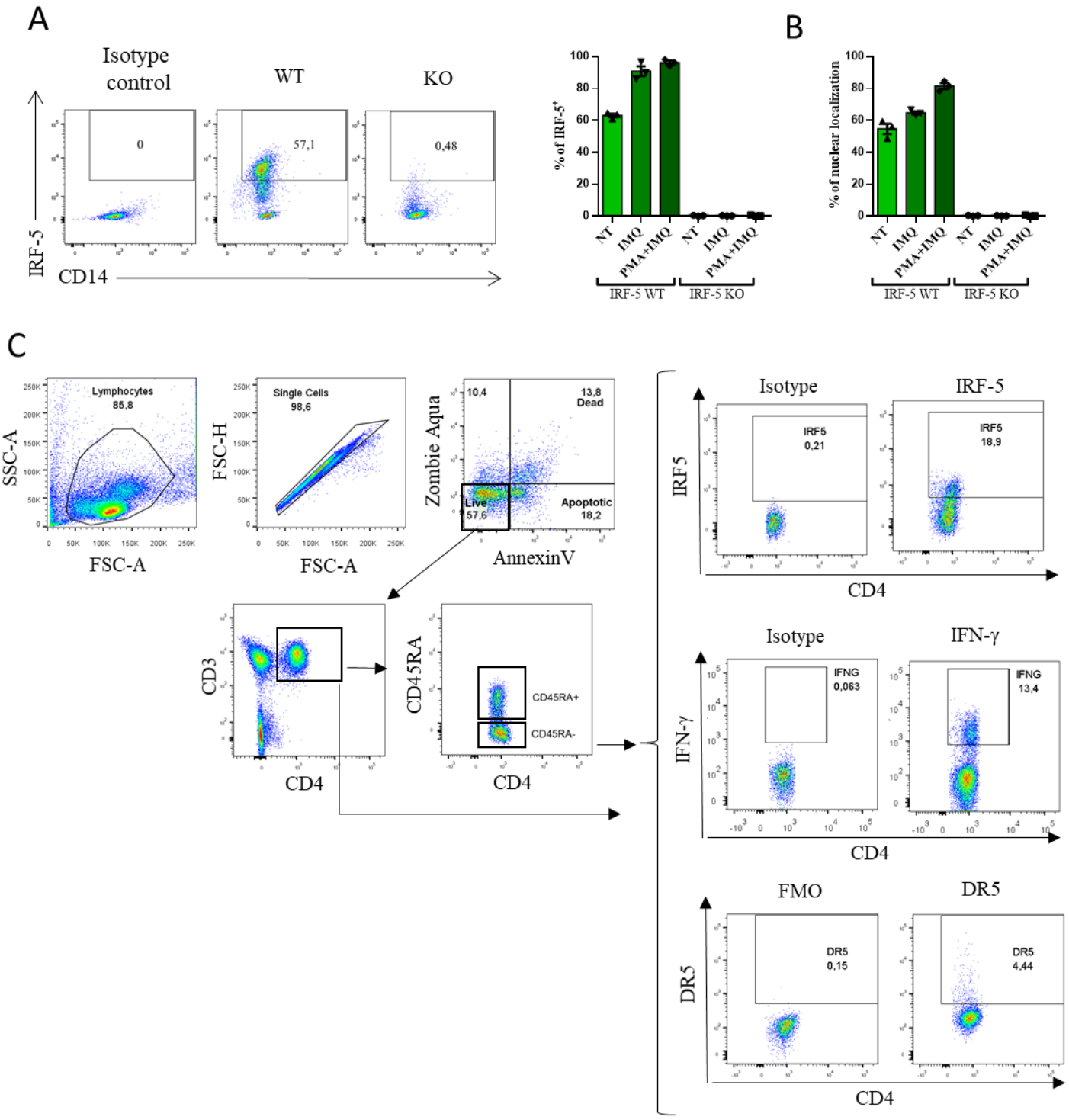
Figure 3.9 summarizes how this novel pathway could work synergistically with other known cell death pathways (reviewed by (Cummins *et al.*, 2010b; Doitsh *et al.*, 2016)), particularly those requiring Caspase 8, to induce apoptosis in $CD4^+$ T cells during primary HIV-1 infection and after ART.

3.6 Acknowledgements

We would like to thank all subjects participating in this study, their physicians, and attending staff members from the Reseau SIDA/Maladies Infectieuses of the Fonds de la Recherche du Quebec-Santé (FRQ-S; Montreal, QC, Canada). We thank Mario Legault and Louise Gilbert for technical and administrative assistance and members of Montreal Primary Infection Study Group. This work was supported by the Canadian Institutes of Health Research (PJT-159647 to S.S.) and the National Sciences and Engineering Research Council (RGPIN-2018-05272 to JvG). LMT and TS were supported by FRQS doctoral awards. J.-P.R is the holder of the Louis Lowenstein Chair in Hematology and Oncology, McGill University.

3.7 Supplemental data

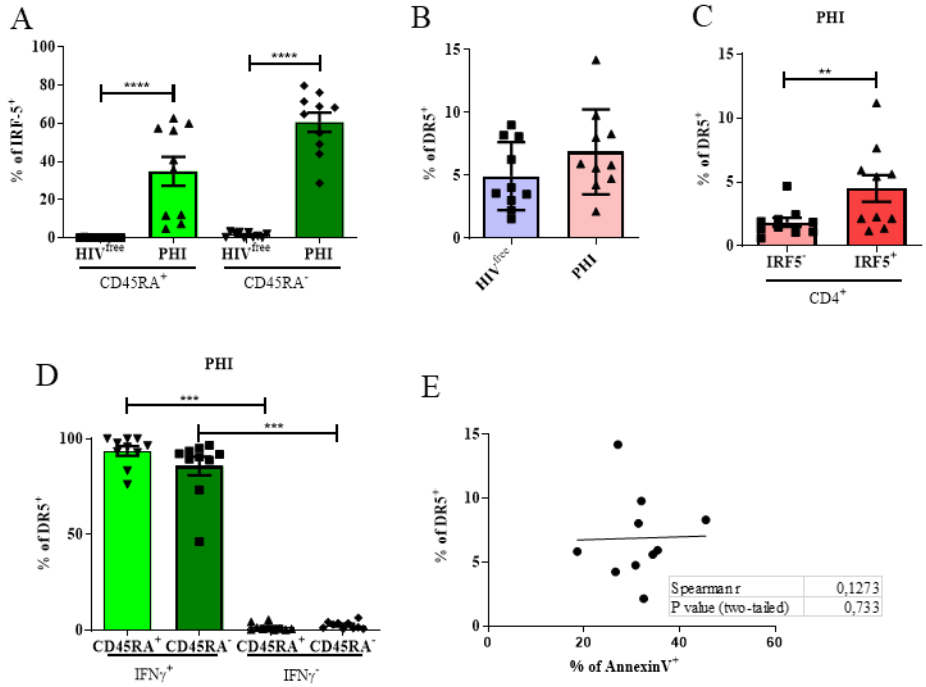
Supplemental Figure 3.10



Supplemental Figure 3.10 Anti-IRF-5 antibody specificity and gating strategy

A) WT and Irf5 $^{-/-}$ THP-1 cells were restimulated with either IMQ or PMA+IMQ and IRF-5 expression was assessed by flow cytometry. Graphs show a) representative FACS plots and the percentage of IRF-5 positive cells, and B) the frequency of IRF-5 nuclear localization. C) Representative FACS plots showing the gating strategy used to determine the frequency of cells positive for Annexin V, IRF-5^{+} , IFNɣ$^{+}$, and DR5^{+} in total CD4^{+} T cells or CD45RA^{+} and CD45RA^{-} CD4^{+} T cells in PBMCs isolated from PHI and HIV^{free} individuals.

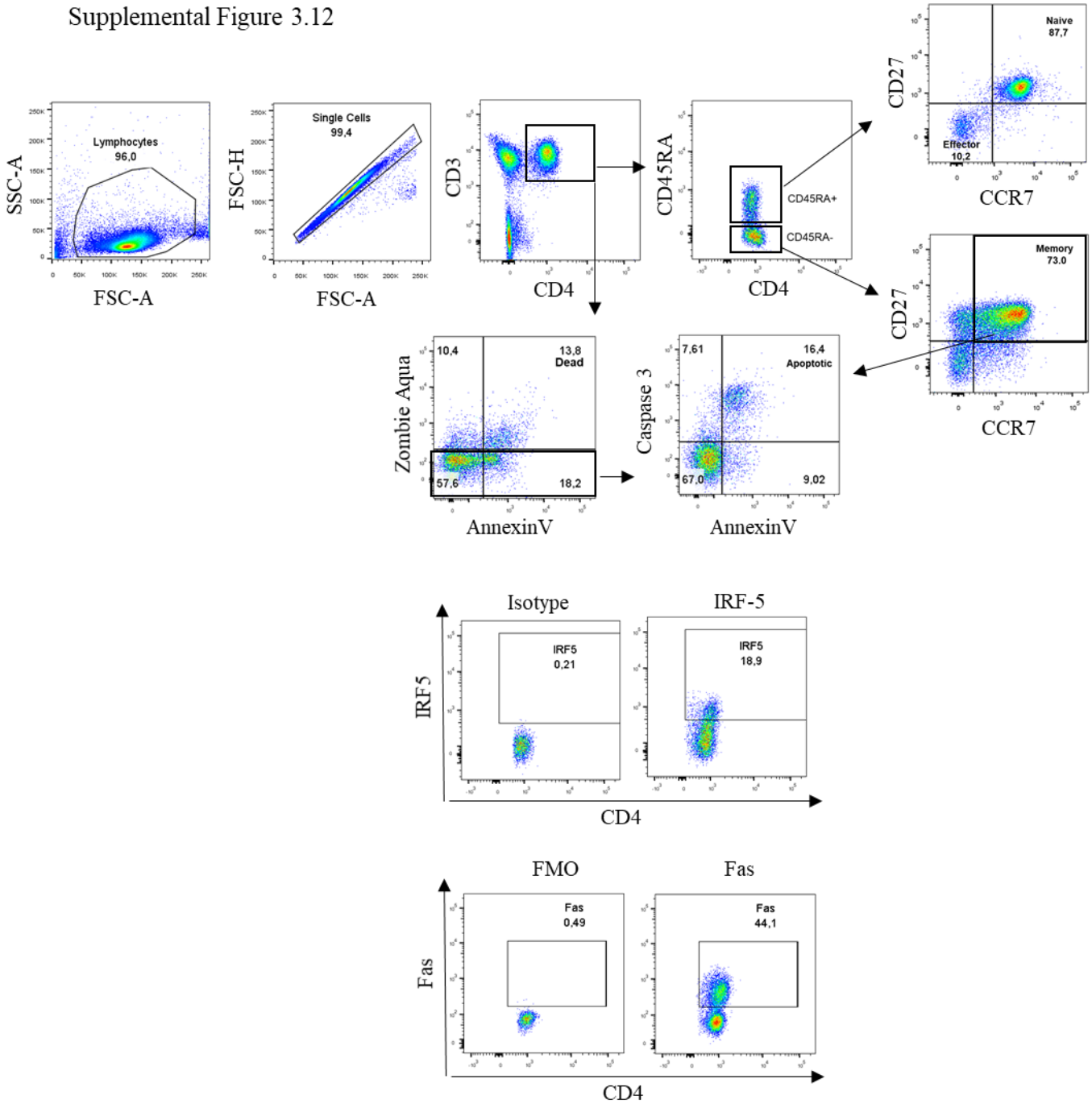
Supplemental Figure 3.11



Supplemental Figure 3.11 DR5 expression in IRF-5^{+} cells

Graphs show a) the percentage of CD45RA^{+} and CD45RA- CD4^{+} T cells expressing IRF-5 and b) the percentage of DR5^{+} CD4^{+} T cells in PHI and HIVfree individuals; c) the percentage of IRF5 expressing DR5^{+} CD4^{+} T cells, and d) the percentage of IRF5^{+} DR5^{+} CD4^{+} T cells expressing CD45RA$^{+/-}$ IFNɣ$^{+/-}$ CD4^{+} T cells in PHI patients. Data are presented as the mean ± SD. The Mann-Whitney U-test and the Kruskal-Wallis test followed by the Dunn's multiple comparison test were used to determine statistical significance, * p < 0.01, *** p

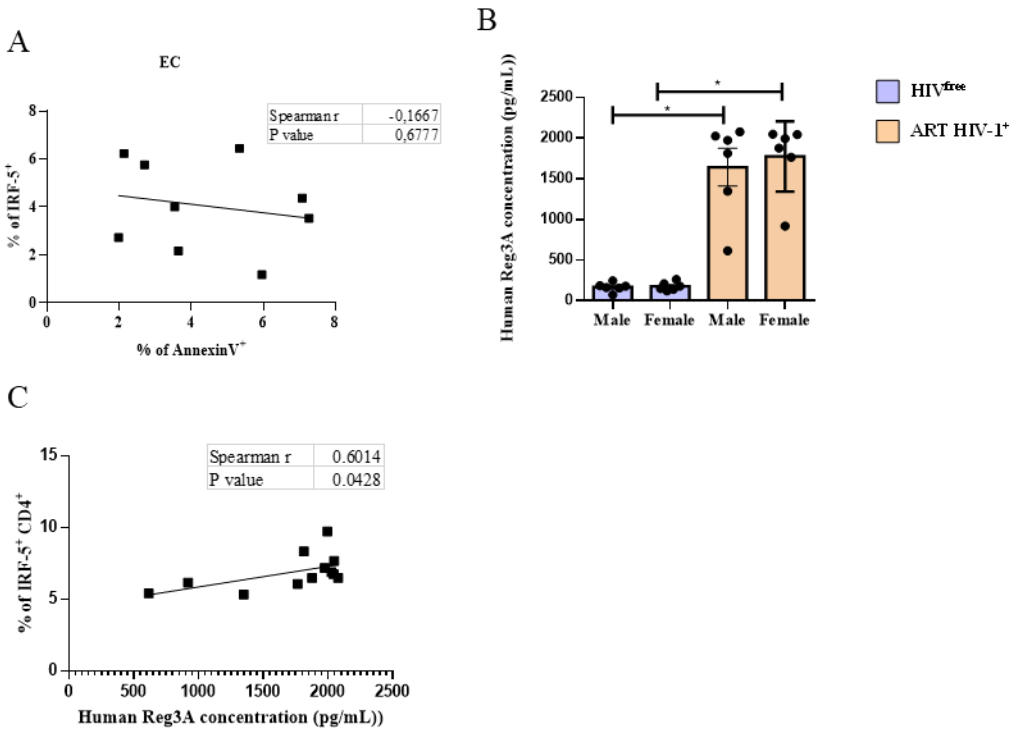
Supplemental Figure 3.12



Supplemental Figure 3.12 Gating strategy

Representative FACS plots showing the gating strategy used to determine the frequency of apoptotic, dead, IRF-5$^+$, and Fas$^+$ CD4$^+$ T cells or to gate naïve, effector and memory CD4$^+$ T cells from ART HIV-1$^+$, EC, and HIVfree donors.

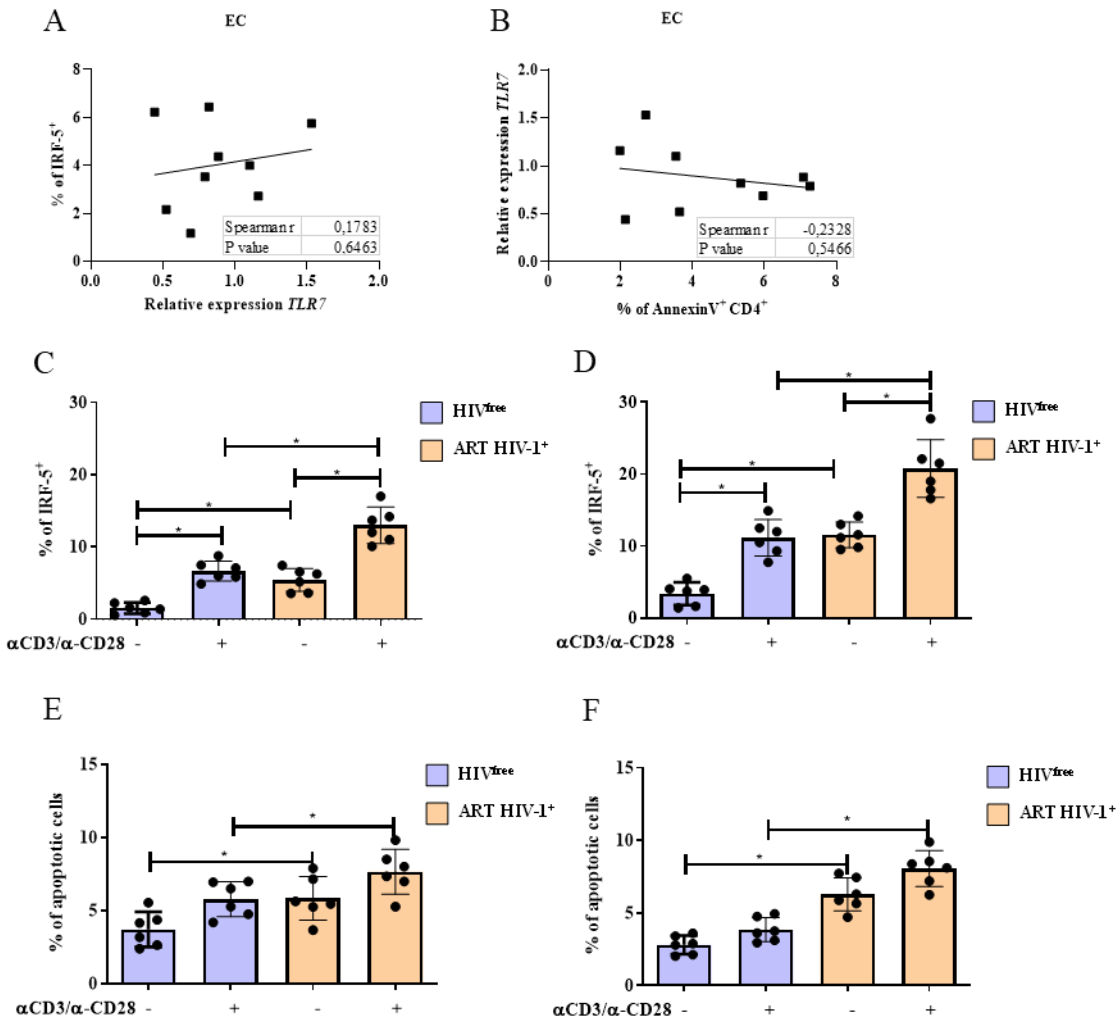
Supplemental Figure 3.13



Supplemental Figure 3.13 IRF-5 expression does not correlate with Annexin V expression in EC but correlates with REG3α concentration in the serum of ART HIV-1$^+$ donors

A) Graph represents correlation between Annexin V and IRF5 expressions in memory CD4$^+$ T cells from EC. The Spearman r test was used to determine statistical significance, * p <0.05, n=10. B-C) The concentration of REG3α in the donors' serum was assessed by ELISA. Graphs show b) REG3α concentrations in the serum of male and female ART HIV-1$^+$ participants, and C) the correlation between IRF-5 expression and REG3α serum concentrations. Data are presented as the mean ± SD. The Wilcoxon test was used to determine statistical significance, * p <0.05, n=6. For C), the Spearman r test was used to determine statistical significance, * p <0.05, n=12 (6 female and 6 male donors).

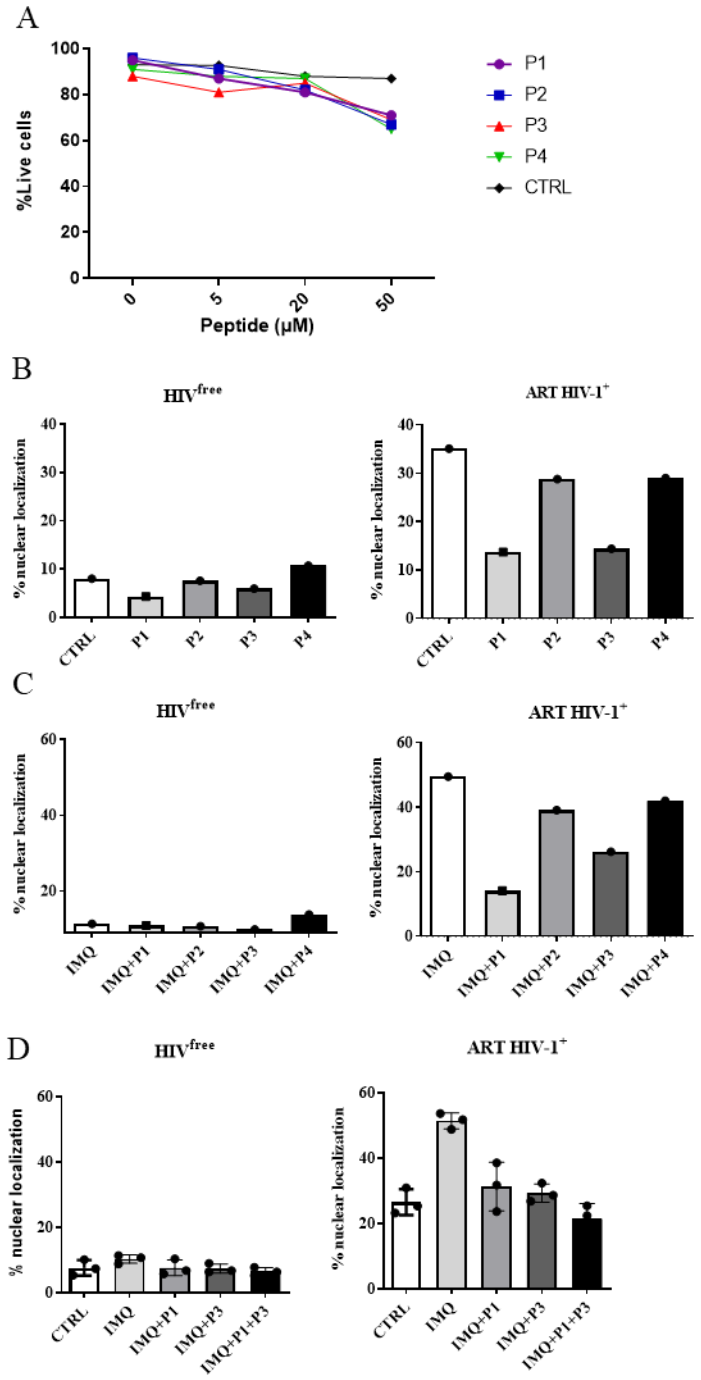
Supplemental Figure 3.14



Supplemental Figure 3.14 Correlations Graphs

Graphs represent correlations between A) *TLR7* and IRF-5 expressions and B) *TLR7* and Annexin V expressions in memory CD4$^+$ T cells from EC. Spearman r test was used to determine statistical differences * p <0.05, n=9. C-F) Purified CD4$^+$ T cells from HIV-1$^+$and HIV^{free} donors were stimulated for 24h with αCD3/αCD28. Graphs show C) the percentage of IRF-5$^+$ total CD4$^+$ T cells and the percentage of d) IRF-5$^+$, e) apoptotic cells, and f) dead cells in memory CD4$^+$ T cells. Data are presented as the mean ± SD. The Wilcoxon test was used to determine statistical significance, * p <0.05, n=6.

Supplemental Figure 3.15

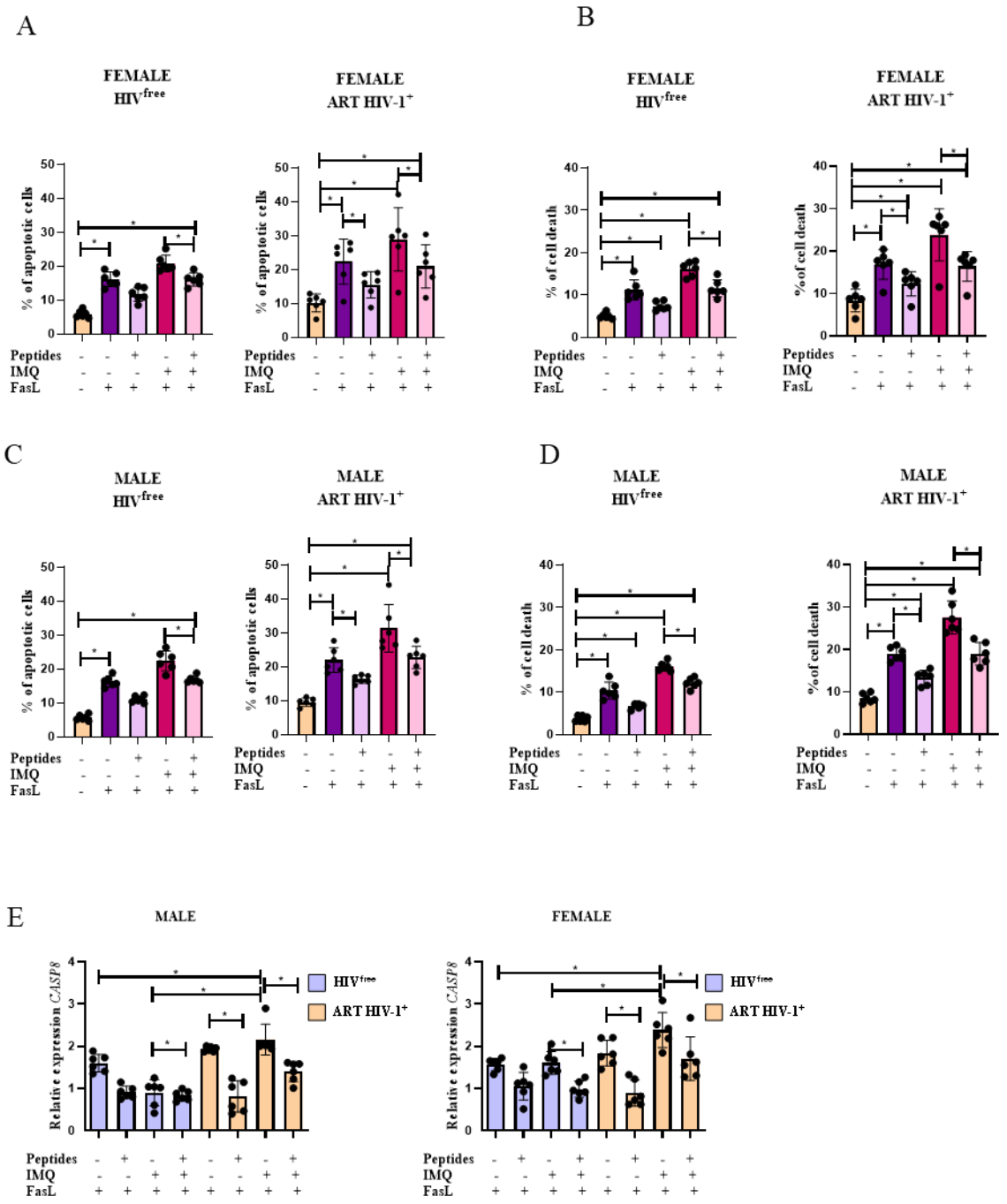


Supplemental Figure 3.15 IRF-5 inhibitory peptides limit Fas-mediated apoptosis in memory CD4⁺ T cells

Four different IRF-5 blocking peptides and the control peptide were tested for their cytotoxicity and their capacity to block IRF-5 in $CD4^+$ T cells. Graphs show A) the percentage of live PBMCs from HIV^{free} donors upon incubation with IRF-5 inhibitory peptides at various concentrations, and B) IRF-5 nuclear localization in unstimulated and C,D) IRF-5

nuclear localization in IMQ-stimulated CD4$^+$ T cells from ART HIV-1$^+$ and HIV^{free} donors in the presence or absence of 10μM of the indicated peptides or peptide mix. Data are presented as the mean ± SD, n=3.

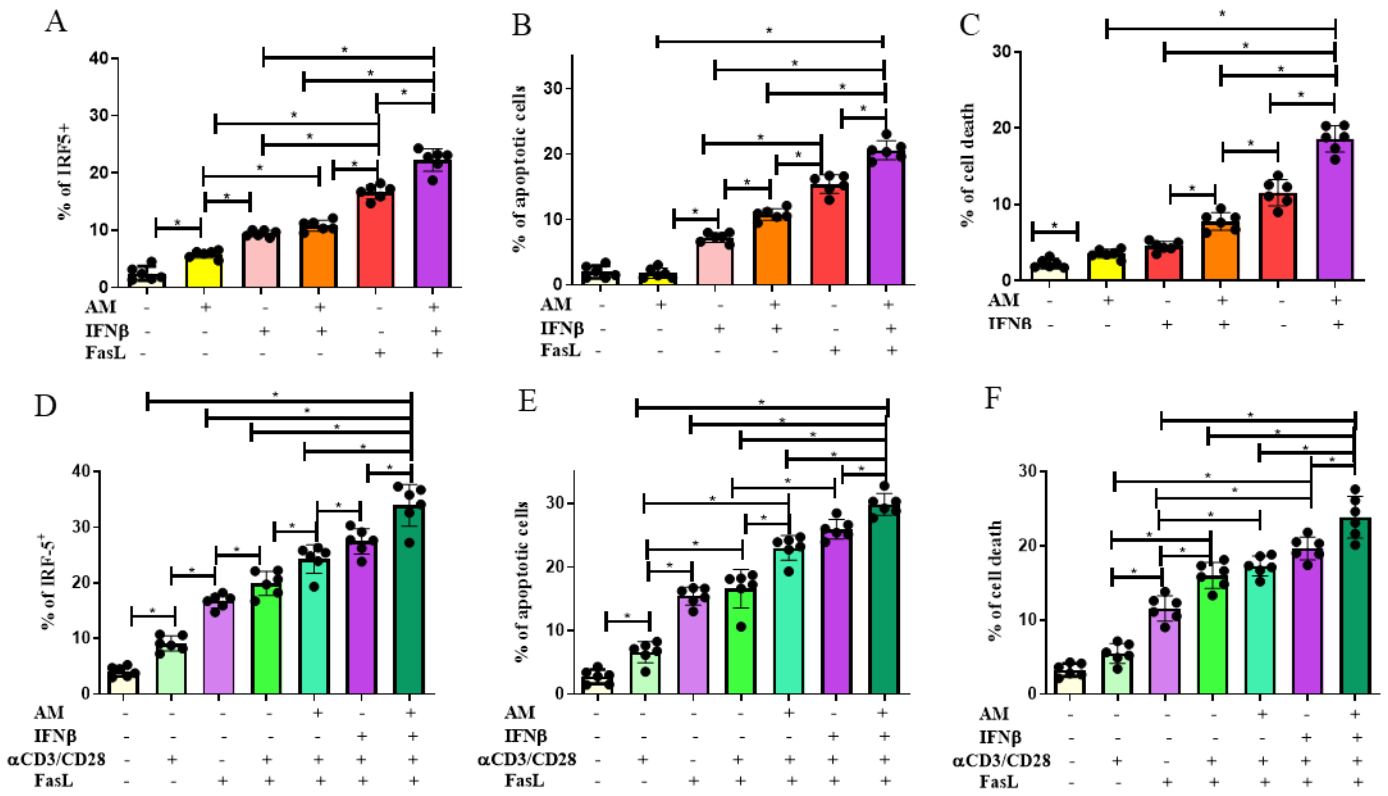
Supplemental Figure 3.16



Supplemental Figure 3.16 CD4 T cells from male and female participants display similar responses

A) Purified CD4⁺ T cells from female and male ART HIV-1⁺ and HIV^{free} donors were treated with IMQ or medium alone; 12h later rFasL or medium were added to the culture and the cells were incubated for further 18h for a total of 30h incubation at 37°C. Graphs show the percentage of apoptotic memory CD4⁺ T cells from A) female and C) male donors , the percentage of dead memory CD4⁺ T cells from B) female and D) male participants, and E) *CASP8* mRNA levels in memory CD4⁺ T cells from male (left graph) and female (right graph) donors after 30h stimulation. Data are presented as the mean ± SD. The Wilcoxon test was used to determine statistical significance, * p <0.05, n=6.

Supplemental Figure 3.17



Supplemental Figure 3.17 Stimulation of memory CD4⁺ T cells in the presence of DAMPs and IFNβ promotes the upregulation of TLR7 and predisposes cells to Fas-mediated apoptosis

Purified CD4⁺ T cells from HIV^{free} individuals were treated *in vitro* for 24 h with 10 ng/ml IFNβ in the presence or absence of 10% v/v supernatant containing apoptotic material (AM, supernatant of staurosporine-treated cells), before stimulation with αCD3/αCD28 for 24h. Graphs show a) the percentage of IRF-5⁺, b) the percentage of apoptotic and c) the percentage of death memory CD4⁺ T cells. CD4⁺ T cells from HIV^{free} individuals were treated as described above and additionally incubated with rFasL for further 18h after αCD3/αCD28 stimulation. Graphs show d) the percentage of IRF-5⁺, e) the percentage of apoptotic and f) the percentage of death memory CD4⁺ T cells. Data are presented as the mean ± SD. The Wilcoxon test was used to determine statistical significance, * p <0.05, n=6.

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4 GENERAL DISCUSSION

Although ART has proven effective in suppressing HIV-1 replication, recovery of a fully functional immune system remains a challenge. PLWH under viral-suppressive ART show varying levels of $CD4^+$ T cell recovery (Yan *et al.*, 2023); however, restoration of $CD4^+$ T cell populations tends to be slow and sometimes incomplete. Indeed, some patients are unable to fully recover their $CD4^+$ T cell count to the level of individuals without health complications. Insufficient recovery of $CD4^+$ T cells despite achieving viral suppression leads to heightened morbidity and mortality (Dronda *et al.*, 2002).

In our efforts to understand the vulnerability of $CD4^+$ T cells to cell death in chronic inflammatory settings, our laboratory recently reported that upregulation of IRF-5 limited $CD4^+$ T cell survival during the chronic stage of experimental VL. Indeed, activation of IRF-5 via TLR7 triggering by inflammatory tissue damage-derived apoptotic cell material induced death receptor 5 and caspase 8, and ultimately led to cell death (Fabié *et al.*, 2018). When we extended our investigation on the TLR7-IRF5 pathway to HIV infection, we found that this axis promotes FAS-mediated apoptosis of memory $CD4^+$ T cells. This mechanism is operative in individuals under ART and may play a role in the maintenance of memory $CD4^+$ T cells and in the recovery of $CD4^+$ T cell counts. Moreover, this pathway may partially explain the poor restoration of the $CD4^+$ T cell population in HIV-infected INRs. More specifically, our findings highlight that memory $CD4^+$ T cells in PLWH on ART express TLR7 and IRF-5 and that this imprint sensitizes them to cell death during chronic infections (Carmona-Pérez *et al.*, 2023).

Different factors could contribute to this imprint on memory cells. Some of these factors are relative to the host polymorphisms, the differential expression of the distinct isoforms of IRF-5, and the level of inflammation, which will determine the presence of DAMPs (Yates *et al.*, 2007).

Throughout this study, many more questions arose that could help to better explain the function of the TLR7-IRF-5 pathway in T cells and the consequences for their maintenance and the immune response. Here are some important points to consider in future studies.

1. IRF-5 and HIV infection

1.1 IRF-5 functions

In this work, we show for the first time that memory $CD4^+$ T cells upregulate IRF-5 in primary HIV-infected donors and PLWH undergoing ART and that this transcription factor is involved in sensitizing memory $CD4^+$ T cells to apoptosis.

The role of IRF-5 in the immune response against viral infections has been previously investigated, but these studies have focused primarily on its function in monocytes, macrophages, or dendritic cells, but not in T cells (Pitha, 2011). In addition, IRF-5 has been poorly studied in the context of HIV infection.

Interestingly, HIV has been shown to interact with IRFs to modulate its activity (Cheng *et al.*, 2014). It could be that the interactions of HIV proteins with IRF-5 alter their transcriptional activity. Although it is possible that, after initiation of antiretroviral therapy, the decrease HIV proteins in $CD4^+$ T cells caused by low viral replication contributes to an increase in the expression of this transcription factor, we do not think that this is the case. Indeed, we believe that the positive regulation of IRF-5 is not determined by viral replication, but it is due to an increase in TLR7 signaling driven by the increase in IFN-I and DAMPs during the chronic phase of the infection. In fact, we think that the cells that have this imprint are not necessarily the infected but are the bystander cells.

Because IRF-5 is not only expressed in memory CD4 T cells, it is important to determine its function in other $CD4^+$ T cell populations to better tailor a potential therapy against HIV infection. For example, IRF-5 may play a role in T cell differentiation and regulation of mucosal inflammation during HIV. Inflammatory bowel disease (IBD) and HIV are characterized by a strong inflammatory environment and tissue disruption especially in the intestinal mucosa. Studies have demonstrated that IRF-5 modulates mucosal inflammation in IBD by controlling Th1 and Th17 immune responses as well as cytokine production. The upregulation of the expression of IRF-5 in $CD4^+$ T cells from individuals with IBD induced their differentiation into Th1 and Th17 cell types by influencing the expression of T-bet and RAR-related orphan receptor C (RORC). IRF-5 overexpression also enhanced Th1/Th17 immune responses, leading to increased secretion of inflammatory cytokines, including IFN-γ, TNF-α, IL-17A, and IL-23. Additionally, IRF-5 expression levels show a significant correlation with disease activity, suggesting its potential as a novel target for therapeutic strategies for this condition (Yang *et al.*, 2021). It would be interesting to investigate whether IRF-5 has a similar function in $CD4^+$ T cells during the acute phase of HIV infection and whether its function changes after ART. It is important to note, though, that T cell-specific *Irf5*$^{-/-}$

mice did not have major defects in their Th1 response against *L. donovani* (Fabié et al., 2018), suggesting that the role of IRF-5 may be tissue and disease-specific.

It is also essential to investigate whether IRF-5 affects HIV reservoirs, as other transcription factors of the IRF family have been reported to affect viral latency. IRF-7 shows a positive correlation with the ability of certain inhibitors, like JAK2 inhibitors, to reactivate latent HIV. This correlation suggests a promising avenue for HIV eradication through innate immune modulation, as it indicates that manipulating IRF-7 levels may contribute to reducing the viral reservoir by activating latent HIV. This approach represents a novel pathway that could be explored further in the quest to eliminate the HIV reservoir from the body (Ezeonwumelu *et al.*, 2022).

IRF-5 is also expressed in Tregs. Hence, another factor to investigate is the role of IRF-5 in $CD4^+$ T cell in INR individuals. It has been shown that this type of $CD4^+$ T cell has alterations in immunoregulation functions involved in the high immune activation existing in INRs. These individuals have a high frequency of Tregs, but with decreased HIV-specific immunosuppressive functions compared with PLWH without ART. However, in INRs, the function of Tregs appears to be primarily associated with regulating the homeostatic proliferation of naïve $CD4^+$ T cells rather than modulating immune activation (Méndez-Lagares *et al.*, 2012).

1.2 IRF-5 isoforms and polymorphisms influence T cell maintenance

Genetic variations in IRF-5 and their association with disease progression in HIV infection has been scarcely studied. These variations could influence IRF-5 expression levels or activity, impacting the host's ability to mount an effective immune response against the virus. Only one report is known related to IRF-5 polymorphisms and immune response to HIV infection. These polymorphisms found in the IRF-5 gene is adjacent to TNOP gene 3, a protein that interacts with the HIV-1 capsid protein (Sepúlveda-Crespo *et al.*, 2023). The IRF5-TNOP3 polymorphisms had significant association with the natural control of HIV infection in EC. It would be interesting to identify whether other IRF-5 polymorphisms that have already been described could be directly related to the predisposition to induce cell death in T cells after ART, which could influence the low maintenance of this cell population in some patients.

Moreover, the function of IRF-5 during HIV infection could be influenced by the type of isoform expressed in T cells. We found in our study that not all peptides designed to block IRF-5 work for this transcription factor in all cell types. This is because T cells do not express the same isoforms as monocytes and dendritic cells. As explained previously, different IRF-5 isoforms are produced

by the alternative splicing that occurs at high rates in the human *IRF5* gene. These isoforms display unique expression profiles in various cell types, demonstrate different subcellular distribution patterns, and fulfill distinct roles in immune responses and the development of diseases (Stone *et al.*, 2013). Remarkably, certain isoforms are associated with an increased risk of SLE, particularly those originating from exon 1B and displaying splice variations within and near the PEST domain located in exon 6. There are several distinctive and consistently produced IRF-5 isoforms that commence from exon 1B, and these isoforms possess the capacity to impact the functionality of IRF-5 or the transcriptional profiles of genes regulated by IRF-5. For example, some of these isoforms are linked to elevated IRF-5 expression and they are linked to a high production of proinflammatory cytokines (Wen *et al.*, 2011).

To date, 16 IRF-5 isoforms have been described, but not all of them are expressed in T cells and the factors inducing differential expression of some isoforms in this specific cell type have not yet been described. It would be of high importance to identify if certain types of isoforms can be linked to the induction of cell death in this type of cells and, even more important, to understand how the expression of these isoforms is triggered (Graham *et al.*, 2006b). It is also crucial to identify which isoform is induced by TLR7 in $CD4^+$ T cells, since a specific isoform could explain the induction of cell death which could be used to design specific peptides only affecting this type of isoform.

To identify what determines the specific function of IRF-5 in $CD4^+$ T cells, it is not only vital to identify the isoforms specifically expressed in $CD4^+$ T cells, but also to identify what type of dimers these isoforms form since the formation of homo- or heterodimers could change their transcriptional targets (Barnes, 2018; Chang Foreman *et al.*, 2012).

1.3 IRF-5 upstream signalling partners

In our study, we explored potential pathways responsible for the upregulation of IRF-5 in $CD4^+$ T cells. We found that the stimulation of TLR7 induced IRF-5 expression in memory $CD4^+$ T cells from ART HIV-1^+.

However, other pathways could be inducing or interacting with IRF-5 during HIV infection. It is known that different pathways synergize to promote inflammatory responses, which can enhance the TLR7/IRF-5 signalling pathway. For example, a study by Yang et al. showed the synergy between CXCL4 and TLR8 to not only enhance TLR8 responses but also skew them toward inflammation (Yang *et al.*, 2022). This is achieved through the combined activation of the TBK1 signaling pathway, which drives inflammatory outcomes by inducing gene expression via IRF-5,

as well as by promoting the production of IL-1β through inflammasome activation. Additionally, this collaboration leads to genome-wide changes in chromatin accessibility. These findings imply that when CXCL4 + TLR8 stimulation occurs, there is a synergistic activation of typical inflammatory genes. This heightened activation of inflammatory pathways may contribute to cytokine storms and the development of inflammatory diseases. However, it also opens up the possibility of therapeutic interventions aimed at selectively dampening pathological inflammation while preserving essential aspects of the host's defense mechanisms.

Additionally, it is essential to identify whether IRF-5 can be induced by other signaling pathways that converge in the induction of cell death through Fas/FasL pathway. For example, whether the reservoir induces the expression of IRF-5 after the reactivation of the infected cells. In these cells it is important to evaluate whether HIV can directly induce IRF-5 through TLR7 or other pathways.

Further research is necessary to fully understand the intricate interactions between IRF-5 and HIV infection. Investigating the specific mechanisms and functional consequences of IRF-5 activation in the context of HIV can provide valuable insights into host-virus interactions and potential therapeutic targets to improve the maintenance of memory cells and immune responses, and to control viral replication in HIV-infected individuals.

1.4 The inhibition of IRF-5 as a possible therapeutic strategy

Because IRF-5 sensitizes memory $CD4^+$ T cell to cell death, IRF-5 inhibition appears as a possible therapeutic strategy. IRF-5 inhibition has mostly been studied in the context of autoimmune diseases. Blocking IRF-5 using conditional *Irf-5* deletion or a novel small-molecule inhibitor has been demonstrated to halt the progression of SLE and effectively sustain remission in mice even after disease onset. These findings imply that inhibiting IRF-5 could potentially address the shortcomings of current SLE treatments, encouraging further exploration of IRF-5 inhibitors in drug discovery research (Ban *et al.*, 2021).

IRF-5 can also be inhibited through the use of peptides. Indeed, IRF-5-inhibiting peptides have been shown to reduce inflammation and fibrosis in the heart and enhance vascular endothelial function in tight skin (Tsk/+) mice (Weihrauch *et al.*, 2016).

Although different strategies have been published to inhibit IRF-5 in mice, the question remains whether these strategies can be extrapolated for therapeutic use in humans. A study utilizing cell-penetrating peptide (CPP) inhibitors, which inhibit IRF-5 activity by targeting homodimerization, points towards the use of IRF-5 inhibiting peptides as a promising therapeutic approach to control

different human diseases in which IRF-5 plays a key role in disease development and progression (Banga *et al.*, 2020).

Thus, strategies of IRF-5 inhibition with demonstrated efficacy in the treatment of diseases such as SLE may also be of interest for treating infections such as HIV. The problem that persists with this approach are potential unintended consequences of blocking IRF-5 in all cell types due to the wide range of functions of this transcription factor. Blocking IRF-5 in general could block $CD4^+$ T cell death, but it could also block other functions of IRF-5 in different cell types, including cytokine and antibody production, differentiation, and proliferation, which are of enormous importance in infection control. Another factor to consider is the different IRF-5 isoforms that are expressed in the various cell types since peptides could be restricted to inhibit only certain types of isoforms and not all of them. Thus, it would be ideal to identify what type of isoform is expressed in memory $CD4^+$ T cells to investigate whether it is specific to a certain cell type and/or to the induction of cell death. In this case, peptides could be designed to regulate only the isoforms of IRF-5 that induces cell death in $CD4^+$ T cells without affecting the other positive functions that IRF-5 may perform in other cell types.

Another strategy to reduce the induction of cell death by IRF-5 is to reduce its activation by inhibiting its phosphorylation, such as, by inhibiting the interaction of IRF-5 with proteins important for its phosphorylation such as TASL (Heinz *et al.*, 2020). However, blocking the interaction of IRF-5 and TALS will inhibit the phosphorylation and activation of all isoforms and thus block other transcription factor functions in T cells and other cell types.

An alternative strategy to regulate IRF-5 expression is to target it for degradation. TRIM21, an E3-ubiquitin ligase, has been shown to facilitate the degradation of IRF-5, offering a potential strategy for modulating IRF-5 levels. However this is isoform-dependent. Lazzari E and collaborators demonstrated that upon activation of TLR7, TRIM21 selectively targets IRF-5 isoforms v1/v5 for degradation, while isoforms v2/v3 remain resistant in macrophages (Lazzari *et al.*, 2014).

The Proteolysis-targeting chimera (PROTAC) technology offers a novel and promising approach to modulate protein levels through induced degradation (Pettersson *et al.*, 2019). This technology could be used for the reduction of overall IRF-5 levels. However, as it does not specifically degrade IRF-5 proteins that only induce cell death, this would not represent a specific blockade of this function.

Another way to control the levels of the death-inducing IRF-5 protein in CD4+ T cells would be to identify whether homodimers and heterodimers define which target molecules can induce death in this cell type and thus block the binding of the death-promoting proteins.

2. TLR7 and HIV infection

2.1 TLR7 functions

In the current study, we have shown that TLR7 is upregulated in memory $CD4^+$ T cells from ART HIV-1^+ individuals. We also have demonstrated for the first time that TLR7 activation in these cells induces IRF-5 expression, which predisposes the cells to Fas-mediated apoptosis.

In contrast to IRF-5, TLR7 has been studied in more detail in the context of HIV. TLR7 has been shown to play a pivotal role during all phases of infection and to have different functions in the various cells of the immune system. The single-stranded RNA of HIV-1 contains numerous uridine-rich sequences that serve as ligands for Toll-like receptors 7 and 8 (TLR7/8). During acute infection, these interactions trigger the activation of plasmacytoid dendritic cells and monocytes, as well as T cell activation mediated by accessory cells. Consequently, TLR ligands encoded by HIV-1 directly contribute to the immune activation observed during acute infection (Meier *et al.*, 2007).

In mice, persistent TLR7 stimulation has been shown to lead to ongoing immune activation, thereby contributing to the gradual decline of immune function and resulting in an immunopathological state akin to the progressive lymphoid destruction seen in HIV infection (Chang *et al.*, 2012). This included lymphopenia, heightened levels of proinflammatory cytokines, splenomegaly, reduced lymphoid cell populations, and structural changes in lymphoid tissues characterized by diminished marginal zone B-lymphocytes (Odermatt *et al.*, 1991). Continuous activation of TLR7 in different cell types has diverse consequences. The finding that TLR7 activation causes B cells to adopt an activated state and release nonspecific antibodies in mice suggests that immunosuppression is not caused by a general inability of B cells to generate IgG (Baenziger *et al.*, 2009). As B-cell activation and hypergammaglobulinemia are hallmark features of HIV infection, the alterations in splenic structure and composition following prolonged TLR7 activation may explain the weakened antigen-specific humoral immune response (De Milito *et al.*, 2004).

In addition, stimulation of TLR7 was found to result in sustained elevation of IL-10, IL-6, IFN-ɣ and TNF levels, reminiscent of the elevated levels observed in chronic HIV infection (Norris *et al.*,

2006). Sustained production of TNF at low levels has significant effects on tissue development, notably leading to reduced thymic tissue. Consistent with these findings, mice treated with R848, a TLR7 agonist, displayed decreased cellularity in the thymus, suggesting that continuous TLR7 stimulation could impair thymic function and its ability to counterbalance the loss of T cells associated with HIV infection (Glosli *et al.*, 2004).

TLR7 signaling also negatively regulates $CD8^+$ T cell responses during HIV-1 infection. Indeed, elevated TLR7 levels in $CD8^+$ T cells among individuals with HIV-1 infection contribute to aberrant immune activation, IFN-ɣ production, and progression of HIV-1 pathogenesis (Song *et al.*, 2009).

It is also known that TLR7 can induce anergy in human $CD4^+$ T cells during HIV acute infection. Reducing TLR7 gene expression using shRNA resulted in a lower occurrence of $CD4^+$ T cells infected with HIV-1 *in vitro* and restored the responsiveness of these HIV-1-positive $CD4^+$ T cells (Dominguez-Villar *et al.*, 2015).

2.2 TLR7 polymorphisms

TLR7 polymorphisms also affect the function of this receptor and increase susceptibility to HIV-1 infection favouring disease progression. These polymorphisms impact the amount of antiviral cytokines, such as IFNs, TNF, interleukin-6 (IL-6), and IL-12, produced after TLR7 stimulation (Zaidane *et al.*, 2020b). For example, the TLR7 polymorphism Gln11Leu has been linked to elevated viral loads, a notable reduction in IFN-α secretion and a hastened progression toward advanced immune suppression in individuals with HIV (Said *et al.*, 2014). Differences in IFN-I levels among individuals are also attributed to the presence of TLR7 polymorphisms. For example, the polymorphism of human TLR7, rs179008, is associated with lower viremia and decreased TLR7-driven IFN-I production by female pDCs (Azar *et al.*, 2020).

Variations in TLR7 genes are associated with susceptibility and disease progression in Chinese MSM (men who have sex with men) individuals infected with HIV-1. Specifically, the TTA haplotype correlates with reduced susceptibility and a slower disease progression during acute infections. Meanwhile, in patients with chronic HIV infection, the minor allele G of TLR7 rs179009 and the haplotype CTG are overrepresented, suggesting they may serve as "risk factors" during this stage of the infection (Zhang *et al.*, 2020). Similarly, the TLR7 SNP (rs2074109) appears to play a role in predisposing individuals to HIV-1 infection in Indian populations (Shaikh *et al.*, 2019).

TLR7 polymorphism could also affect the sustained TLR7 activation and the imprint that predisposes memory $CD4^+$ T cells to Fas-mediated apoptosis. It would be interesting to

investigate whether certain TLR7 polymorphisms are associated with the activation of IRF-5 and whether patients that do not respond to therapy and do not regain CD4 counts have one of these TLR7 polymorphisms.

2.3 Induction of TLR7 expression

Key questions in our study pertained to the function of TLR7 in $CD4^+$ T cells during HIV infection and the induction and maintenance of TLR7 expression even after ART. Our investigation revealed a synergistic effect between IFN-β and DAMPs in the induction of TLR7 expression in these cells. This synergistic effect was further amplified by TCR signaling. These findings suggest that the intensity of the IFN-I response, immune activation, and inflammation-derived tissue damage could influence the degree of TLR7 expression on $CD4^+$ T cells, including both antigen-specific and bystander cells. In autoimmunity models, the level of IFN-β has been shown to contribute to TLR7 induction in other cell types, creating a feedback loop in which IFN-β production is dependent on TLR7 activation and TLR7 overexpression is dependent on the IFN-I response (Green *et al.*, 2009b). This could explain how TLR7 expression in $CD4^+$ T cells is maintained over time.

The role that IFN-I play during HIV antiviral responses are complex. IFN-I is essential for triggering antiviral responses during the acute phase, but persistent IFN-I signaling amplifies immune activation promoting immune exhaustion, and enhances the expression of various inhibitory factors, which suppress antiviral immunity, contributing to viral shedding and the establishment of persistently infected reservoirs (Marsden *et al.*, 2012). Our results demonstrate that IFN-I, in addition to inducing chronic immune activation and formation of reservoirs, also induces TLR7 expression leading to the development of an imprint that predisposes memory $CD4^+$ T cells to cell death. Additionally, it has been proposed that the combination of ART with IFNR blockade during chronic HIV infection would accelerate viral suppression and reduce the reactivatable reservoir of HIV (Zhen *et al.*, 2017). It would be interesting to study the impact of blocking IFN-I signaling on the activation of the TLR7 signaling pathway and the induction of Fas-mediated apoptosis.

Yet, IFN-I is not sufficient to perpetuate TLR7 expression and create this imprint. Despite effective ART, PLWH continue to experience persistent chronic immune activation and inflammation. Hence other factors may determine the levels of immune activation and inflammation after ART. These factors may impact T cell activation through the TCR and the availability of DAMPS that are also necessary to perpetuate TLR7 expression and activation and induce cell death. The

inflammatory state that persists after ART is attributed to multiple factors, including thymic dysfunction, persistent antigenic stimulation due to low residual viremia, microbial translocation and dysbiosis due to intestinal mucosal disruption, co-infections, and cumulative ART toxicity (Zicari *et al.*, 2019). In our study, we have not yet determined which specific DAMPS promote TLR7 expression. We propose that the source of these DAMPS may be microbial translocation and dysbiosis due to altered intestinal mucosa. Finding ways to decrease IFN-I signaling, immune activation and chronic inflammation at the time of ART initiation could educe TLR7 expression levels in memory cells and ultimately reduce their predisposition to cell death.

2.4 TLR7 activation

Our results highlight that TLR7 is possibly activated by ligands from microbial translocation and not by reactivation of viral replication in individuals on ART.

After the commencement of ART, the rapid decrease in immune activation correlates closely with the decrease in HIV-1 viremia. This implies a direct involvement of HIV-1 in immune activation, in which TLR7 is implicated (Meier *et al.*, 2007). However, after receiving ART, people living with HIV still exhibit persistent low-grade immune activation and inflammation. In this scenario, TLR7 triggering is not directly activated by HIV-derived ligands. The specific ligand inducing TLR7 activation in uninfected or not HIV ssRNA-exposed cells during ART remains to be identified. We hypothesize that they could be DAMPS derived from tissue damage due to inflammation and microbial translocation from the damaged gut.

The gut mucosa and GALT play a vital role in maintaining overall physiology and health. HIV specifically targets mucosal $CD4^+$ T cells, causes their decline within the gut, leading to significant disruptions in gut homeostasis. The virus also causes increased epithelial cell death and reduced cell-cell adhesion within the gut, altering the microbial balance and allowing microbial products to enter the bloodstream, leading to dysbiosis (Zevin *et al.*, 2016). These disruptions persist despite ART, leading to prolonged immune activation and inflammation, which can be compounded by opportunistic co-infections (Massanella *et al.*, 2016). These microbial products could act as DAMPS that activate different TLRs such as TLR7.

To assess the level of gut integrity, circulating markers have been utilized, particularly in PLWH. Intestinal fatty acid-binding protein (I-FABP), released into the bloodstream upon gut epithelium cell death, has been identified as a key marker in this context. Numerous studies have consistently shown elevated I-FABP blood levels in PLWH, irrespective of their use of ART, in comparison

with individuals without HIV. Additionally, regenerating islet-derived protein 3 α (REG3α), an antimicrobial peptide typically produced in the gut lumen, undergoes translocation to the submucosa in response to gut damage and the detection of REG3α in the circulation serves as an indicator of increased gut permeability, providing valuable insights into the impact of HIV on gut health (Ouyang *et al.*, 2023). Indeed, higher levels of REG3α have been observed in PLWH, regardless of ART usage, when compared to HIV-negative controls (Isnard *et al.*, 2020a).

Gut damage associated with HIV facilitates the entry of microbial products into the submucosa and systemic circulation. This translocation is often identified by measuring the levels of bacterial LPS in the blood. Elevated LPS levels have been observed in both individuals with untreated HIV and those undergoing ART. Additionally, the translocation of fungal products such as β-D-glucan (BDG) has been documented in HIV infection, and elevated BDG levels persist in PLWH receiving ART (Ramendra *et al.*, 2019; Xun *et al.*, 2022).

In our study, we observed a notable increase in REG3α levels in ART-treated HIV^+ individuals, contrasting the minimal levels detected in HIV-free donors. Additionally, a noteworthy correlation emerged between IRF-5 expression frequency and REG3α levels in the serum of ART-treated PLWH. These findings suggest a potential link between these DAMPs released from compromised intestinal mucosa, TLR7 activation, and IRF-5 induction (Carmona-Pérez *et al.*, 2023).

Heightened immune activation has shown a connection to microbial translocation in both untreated and treated HIV infection scenarios (Cassol *et al.*, 2010). Indeed, innate immune activation, markers of inflammation, barrier dysfunction as measured by soluble markers predict higher mortality rates in individuals undergoing treatment for HIV infection (Hunt *et al.*, 2014). Multiple studies have linked gut damage and the translocation of microbial products into the bloodstream to inflammation and an elevated risk of non-AIDS-related comorbidities in PLWH undergoing ART. These comorbidities include conditions like adiposity, cardiopulmonary issues, and neurocognitive impairment (Dirajlal-Fargo *et al.*, 2019; Gianella *et al.*, 2019; Morris *et al.*, 2012).

As shown in this project, DAMPS derived from tissue damage activate the TLR7/IRF-5 axis which induces $CD4^+$ T cell death in the absence of viral replication. This could explain why memory $CD4^+$ T cells remain susceptible to apoptosis triggered by the Fas pathway, even after years of viral suppression in the bloodstream (Carmona-Pérez *et al.*, 2023). Other studies also reported that microbial TLR agonists can induce cell death. Funderburg and colleagues have suggested that the immune dysfunction observed in chronic HIV infection could be driven by systemic exposure

to microbial TLR agonists, bacterial LPS, and DNA translocated from the damaged gut. Exposure to TLR ligands has been shown to increase immune activation, cause sequestration in lymphoid tissues and increase T-cell turnover, which aggravates immune dysfunction and contribute to the depletion of $CD4^+$ T cells in chronic HIV infection (Funderburg *et al.*, 2008). In addition, exposing PBMCs to various microbial TLR agonists *in vitro* primarily activates T cells of central memory and effector memory phenotype. $CD8^+$ effector memory cells upregulate CD69 expression, possibly enhancing their retention in secondary lymphoid tissues, while $CD4^+$ central and effector memory T cells are induced to enter the cell cycle and undergo apoptosis (Caron *et al.*, 2005).

Since ART does not fully control microbial translocation, which is linked to inefficient $CD4^+$ T cell reconstitution, it would be interesting to evaluate whether the TLR7/IRF-5 signaling pathway is active in INRs individuals. It has been shown that failure to regenerate the $CD4^+$ T cell pool in INRs is related to the polymicrobial flora in peripheral blood that remains largely unaltered by therapy. It might be intriguing to analyze whether the induction of $CD4^+$ T cell death in these individuals is caused by the TLR7/IRF-5 imprint that predisposes these cells to Fas-mediated apoptosis (Merlini *et al.*, 2011). However, other factors that may influence reconstitution in these individuals should be considered. A swifter rebound in $CD4^+$ T cell counts post-antiretroviral therapy is also linked with initial factors such as a higher baseline $CD4^+$ T cell count, younger age, reduced pre-ART LPS level, elevated pre-ART soluble CD14 level, diminished pre-ART IL-7 level, and IL-7R ha (Rajasuriar *et al.*, 2010).

Although we strongly believe that the TLR7/IRF-5 imprinting predisposes memory cells to Fas-mediated apoptosis, it is appropriate to evaluate other pathways of cell death caused by microbial translocation. In pathogenic SIV infection, findings suggest that intestinal barrier dysfunction leads to microbial translocation, driven by significant neutrophil infiltration, heightened epithelial proliferative activity, and sustained gut mucosal apoptosis (Estes *et al.*, 2010). Neutrophil invasion and mucosal cell death remain persistently elevated even with ART. Although epithelial growth increases in HIV, it might be hindered in individuals with a poor immune response. Whether mucosal cell death causes, or results from, defects in epithelial growth remains uncertain but seems linked to widespread inflammation in the body. Future studies are warranted to explore the effects of ART and methods that focus on enhancing the gut's protective lining in treated HIV cases (Somsouk *et al.*, 2015).

2.5 TLR7 pathway during HIV infection

Our study identified two downstream targets of TLR7 signaling in memory $CD4^+$ T cells: IRF-5 and caspase-8. However, TLR7-IRF-5 axis could have also other downstream signalling partners. For instance, the TLR7-induced production of IFN-β in human plasmacytoid dendritic cells has been described, which requires direct phosphorylation of IRF-5 by IKKβ for it activation following ligation of TLR7 (Hayden *et al.*, 2014). Other transcription factors could also be induced by TLR7 during HIV infection in $CD4^+$ T cells, which may contribute to the induction of cell death. For example, TLR7 signaling can trigger NF-κB-dependent and IRF7-dependent pathways, leading to the activation of innate immune responses and the production of IFNs (Trinchieri *et al.*, 2007).

In addition, various pathways involving TLR7 may have a synergistic effect on the induction of lymphopenia, which could be the case for the TLR7/IRF-5 axis as the IFNAR and IRF7-dependent pathways cannot completely account for the lymphoid system disruption. Although effects upon TLR7 triggering in *Ifnar*$^{-/-}$ or *Irf7*$^{-/-}$ mice were generally less pronounced, these mice still presented with lymphopenia, demonstrating that TLR7 can cause lymphocyte depletion by other IRF-7-independent mediated mechanisms, which may include IRF-5 (Baenziger *et al.*, 2009; Kamphuis *et al.*, 2006).

2.6 Inhibition of the TLR pathway

One strategy to curb the chronic immune activation instigated by HIV-1 and its accompanying immune pathogenesis is to impede the TLR7 pathway directly. Blocking the TLR7 pathway is now acknowledged as a crucial mechanism for regulating IRF-5 function. However, TLR7 signaling is also important in the control of infection and completely blocking TLR7 in all cell types would also have negative consequences. For example, TLR7 contributes to intracellular defense against HIV in macrophages, as TLR7 agonists such as imiquimod can inhibit viral entry by boosting the production of CC chemokines like MIP-1α, MIP-1β, and RANTES, which are natural ligands for the HIV entry co-receptor CCR5. The expression of CCR5, as well as CD4, can also be reduced after triggering TLR7 (Meng *et al.*, 2021).

In addition, blockade of TLR7 could reduce the activation of HIV-1-specific memory T cell responses, because TLR7 activation in specific subsets of dendritic cells may amplify their ability to activate virus-specific T cells (Hornung *et al.*, 2005; Loré *et al.*, 2003). Hence, inhibition of TLR7 is definitely not the best strategy to prevent memory cell death because of the numerous adverse effects this will cause.

2.7 The therapeutic potential of TLR7 agonists

TLR7 agonists hold significant therapeutic potential in HIV infection by reducing viral reservoirs, enhancing antiviral immunity, and possibly contributing to strategies aimed at achieving long-term remission or functional cure. However, ongoing research is essential to fully understand their efficacy and safety profiles (Zheng *et al.*, 2023).

Activation of TLR7 has contrasting effects during acute versus latent HIV infection. In fact, stimulating TLR7/8 could offer a potential avenue for clearing latent HIV without inducing rapid cell death, thus maintaining a controlled replication level while preventing new infections (Schlaepfer *et al.*, 2006). Different TLR7 agonists have been explored as possible HIV latency reversal agents to support ART therapies and as possible adjuvants to include in vaccine design (Sartorius *et al.*, 2021). For example, TLR2 and TLR7 agonists, when used in combination, efficiently revive dormant HIV by stimulating immune activity and fostering viral reactivation within $CD4^+$ T cells. A similar activation of the virus is observed when triggering TLR7 using its agonist vestatolimod (GS-9620) in monocytes and plasmacytoid dendritic cells, which results in the release of TNF (Macedo *et al.*, 2018).

Co-administering TLR7/8 and TLR9 agonists in an adjuvant formulation substantially boosts antibody responses against the HIV-1 envelope in Rhesus macaques, suggesting potential advantages for forthcoming vaccines. Incorporating TLR7/8 and TLR9 agonists into a squalene-based oil-in-water emulsion enhances the generation of HIV-1 antibodies. This adjuvant combination preserves the antigenicity of recombinant HIV-1 Envs and is expected to be a robust formulation to pair with highly antigenic Env capable of eliciting substantial levels of potentially protective antibodies (Moody *et al.*, 2014). Such TLR7 agonists, when used alongside other treatments, prevent viral rebound following antiretroviral therapy discontinuation and have demonstrated efficacy in maintaining long-term control of SHIV or SIV in nonhuman primates models (Borducchi *et al.*, 2018; Moldt *et al.*, 2022).

Additionally, the TLR7 agonist vesatolimod has demonstrated the ability to activate HIV in PBMCs from individuals with HIV who are on ART. It also reduces viral replication by stimulating the production of IFN-α (Bam *et al.*, 2017; Tsai *et al.*, 2017). Vesatolimod has been shown to be well tolerated and was found to induce immune activation in adults living with HIV-1. It also shows promising results in modestly delaying viral rebound in HIV controllers following cessation of

antiretroviral therapy, which supports the need for future combination trials (Riddler *et al.*, 2021; SenGupta *et al.*, 2021).

TLR agonist-based therapy has even been attempted as a preventive treatment. Exposure to TLR stimulation in HIV-1-exposed seronegative individuals triggers a heightened release of immunological factors, potentially enhancing adaptive antiviral immune responses. This phenomenon represents an innate immune protective phenotype induced by virus exposure against HIV-1 (Biasin *et al.*, 2010). However, it is important to consider when the therapy should be administered and its specific effects on each cell type when designing a new therapeutic approach. During acute HIV infection, the activation of TLR7/8 may create an antiviral environment that limits excessive HIV replication. Conversely, TLR7/8 activation in latently infected cells may prompt the release of HIV virions (Schlaepfer *et al.*, 2006).

Although therapies based on TLR7 agonists help myeloid cell activation and promote the elimination of the virus during the onset of the infection, it is important to consider that TLR7 activations could negatively impact memory $CD4^+$ T cells during the chronic phase of the disease by inducing cell death in these cells. This could also affect effector $CD4^+$ T cells whose elimination will increase the probability of developing AIDS, especially in PLWH who do not respond to ART.

3 Fas and HIV infection

3.1 Fas-induced apoptosis

Fas and Fas ligand have been identified as playing a vital role in the management of cell death within the immune system, particularly in regulating the balance and proliferation of T lymphocytes. The Fas/FasL pathway is involved in immune cell depletion in HIV infection, in which the virus directly or indirectly triggers the loss of $CD4^+$ T cells.

A prominent theory explaining cell depletion in HIV infection revolves around apoptosis, as activating the Fas pathway is considered the primary apoptotic mechanism. Naïve T cells in the periphery exhibit minimal or no cell surface Fas expression, while activated memory T cells display relatively higher levels of Fas. This higher Fas presence in memory cells is associated with their increased susceptibility to apoptosis, emphasizing the role of Fas in immune system homeostasis (Salmon *et al.*, 1994; van Grevenynghe *et al.*, 2008c).

During acute HIV infection, there is a prolonged disruption in the Fas/FasL pathway which may not solely relate to virus production but could play a role in disease progression. One hypothesis suggests a delicate balance between proapoptotic events (upregulation of Fas expression),

possibly initiated by the host to restrict viral replication, and antiapoptotic events (downregulation of FasL expression), potentially instigated by the virus to enhance its replication and persistence (Cossarizza *et al.*, 2000; Poonia *et al.*, 2009).

Our study proposes a model where Fas-mediated apoptosis is largely enhanced by the imprint of TLR7 and IRF-5 expression in memory $CD4^+$ T cells during HIV infection, highlighting how two cell death-promoting signaling pathways may synergistically interplay to reduce $CD4^+$ T cell count. One of the most important perspectives of our study is to explore whether the TLR7/IRF-5 pathway is active and feeding into the Fas/FasL pathway in INR individuals which could explain the poor restoration of $CD4^+$ T cell counts in these individuals. Although the Fas/FasL pathway is one of the most important in cellular depletion in HIV infection, we must keep in mind that other cell death signaling pathways and other factors may contribute to the low $CD4^+$ T cell count in INRs. For example, auto-IgG binding on $CD4^+$ T cell surfaces and its mediation of $CD4^+$ T cell death via NK cell cytotoxicity is associated with elevated $CD4^+$ T cell apoptosis and impaired $CD4^+$ T cell recovery in HIV^+ subjects on ART with viral suppression (Luo *et al.*, 2017).

3.2 Induction of Fas/FasL pathway

Our study suggests that IRF-5 is the key that links TLR7/IRF-5 and the Fas/FasL pathway. We demonstrate that the TLR7/IRF-5 axis is essential for the sensitization of memory cells to Fas-mediated apoptosis. Our results are supported by previously published findings that demonstrate that IRF-5 is essential for Fas-mediated apoptosis (Couzinet et al., 2008).

However, as for IRF-5 and TLR7, genetic factors such as polymorphisms may affect the expression of Fas and FasL and thus the induction of apoptosis via this pathway. Two polymorphisms in the 5′-flanking region of the human Fas gene have been identified (Huang *et al.*, 1997). The first polymorphism is located 670 bp upstream from the gene's translational start site, at the binding site for signal transducers and activators of transcription (STAT) molecules (Huang *et al.*, 1999). The second polymorphism is a G to A substitution at position −1377 relative to the translational start site. Although this change does not create or remove any restriction site, it abolishes the consensus sequence of the binding transcription factor SP-1 (Huang *et al.*, 2000). As for FasL, two polymorphic sites have been identified in the second and third introns of the gene (Bolstad *et al.*, 2000; Pinti *et al.*, 2002). The first polymorphism, IVS2nt-124, involves an A to G substitution at the nucleotide 124 positions upstream from the first base of the third exon.

The second polymorphism, IVS3nt169, is a deletion of a T at position 169 downstream from the last base of the third exon. The presence of specific Fas or FasL polymorphisms have a significant impact on $CD4^+$ T cell generation and the reduction of viral load. These genetic variations appear to influence the immune recovery process initiated by antiretroviral therapy (Nasi *et al.*, 2005).

Another factor that can influence induction of Fas/FasL pathway is the activation of transcription factor Foxo3a. Genes involved in the extrinsic apoptotic pathway, such as Fas ligand and TRAIL, or components of the intrinsic apoptotic pathway genes, such as PUMA, Noxa, and Bim are targets of Foxo3a (Dabrowska *et al.*, 2008). Foxo3a affects the generation and maintenance of lymphocyte memory (Riou *et al.*, 2007; van Grevenynghe *et al.*, 2008a). The reduced transcriptional activity of Foxo3a in ECs increased survival of memory cells, it serves as a molecular signature or indicator of immunity, linked to natural protection and the absence of disease progression (van Grevenynghe *et al.*, 2008b). In the context of INRs, it would also be interesting to confirm whether cells that express FOXO3 are those that directly induce cell death in memory cells that express TLR7-IRF-5, contributing to the impairment of the generation and maintenance of memory of the lymphocytes (van Grevenynghe *et al.*, 2008a).

3.3 Downstream target of Fas signaling

It is established that Fas signaling serves a dual purpose by inducing both apoptosis and the production of inflammatory cytokines. Specifically, among the cytokines elicited by Fas activation, the IL-1β family requires proteolytic processing for their biological activity. In response to pathogens and danger signals, inflammasomes are activated and cleave IL-1β cytokines through caspase-1. Interestingly, the activation of Toll-like receptors (TLRs) leads to an upregulation of Fas, making them responsive to Fas ligand-receptor engagement. In macrophages and dendritic cells, Fas signaling activates caspase-8, resulting in the maturation of IL-1β and IL-18 independently of inflammasomes or RIP3, thus controlling a unique, noncanonical pathway for IL-1β activation in myeloid cells which potentially plays a crucial role in inflammatory processes (Bossaller *et al.*, 2012). It would be interesting to investigate whether Fas signaling is involved in the production of inflammatory cytokines during HIV infection. It may also contribute to the inflammatory environment that increases the expression of TLR7 and IRF-5 in $CD4^+$ T cells.

3.4 The inhibition of IRF-5 as a possible therapeutic strategy

We and others showed that memory $CD4^+$ T cells have an increased susceptibility to Fas-mediated apoptosis (Carmona-Pérez *et al.*, 2023; van Grevenynghe *et al.*, 2008c). It has been shown that a large proportion of T cell death in HIV-infected individuals is due to Fas FasL-mediated apoptosis and has been proposed that cysteine protease inhibitors can be used to prevent Fas ligand up-regulation in HIV-infected patients to decrease apoptosis (Yang *et al.*, 1997). However, completely blocking Fas-mediated apoptosis would affect the natural balance between cell death and proliferation, as well as prevent the elimination of reservoir or infected cells by other infections.

Therefore, we propose a more specific blockade of memory $CD4^+$ T cell death using peptides that inhibit IRF-5 activation and thereby decrease the activation of TLR7/IRF-5 and the Fas/FasL pathway.

In conclusion, the TLR7/IRF-5 axis plays a significant role in the pathogenesis of HIV-1 infection by sensitizing memory $CD4^+$ T cells to Fas-mediated apoptosis. Understanding the TLR7/IRF-5 axis opens up potential therapeutic avenues. By targeting this pathway, it might be possible to reduce the destruction of memory $CD4^+$ T cells, thereby preserving immune function and improving the health outcomes of PLWH.

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6 APPENDIX I

List of publications from collaborations

1. Swaminathan S, Mai LT, Meli AP, Carmona-Pérez L, Charpentier T, Lamarre A, King IL, Stäger S. LAG-3- and CXCR5-expressing CD4 T cells display progenitor-like properties during chronic visceral leishmaniasis. Cell Rep. 2024 Mar 26;43(3):113879. doi: 10.1016/j.celrep.2024.113879. Epub 2024 Feb 27. PMID: 38416647.

2. Stögerer T, Silva-Barrios S, Carmona-Pérez L, Swaminathan S, Mai LT, Leroux LP, Jaramillo M, Descoteaux A, Stäger S. Leishmania donovani Exploits Tunneling Nanotubes for Dissemination and Propagation of B Cell Activation. Microbiol Spectr. 2023 Aug 17;11(4):e0509622. doi: 10.1128/spectrum.05096-22. Epub 2023 Jul 5. PMID: 37404188; PMCID: PMC10434010.

List of Scholarships and awards

1. Prix Perron-Desrosiers highlighting the excellence of the research work, INRS- CAFSB. Laval, Canada. CAD 500.

2. Montreal Immunology Meetings (MIM) travel award for the annual Canadian Society for Immunology (CSI) meeting, Orford, Quebec, CAD 500.

3. The TD Insurance Scholarship Program for the annual CSI meeting, Halifax, Nova Scotia, CAD 500.

List of conference presentations

1. **Canadian Society for Immunology (CSI), 36th Annual Spring Meeting - April 22 to 25, 2024. The Banff Centre, Banff, Alberta, Canada**
   Carmona-Pérez L, Dagenais-Lussier X, van Grevenynghe J, and Stäger S
   "IRF-5 and CD4 T cell death during persistent infection"

2. **Canadian Society for Immunology (CSI), 35th Annual Spring Meeting (National Academic Event, poster presentation), June 6 to 9, 2023. Orford, Québec, Canada**
   Carmona-Pérez L, Dagenais-Lussier X, van Grevenynghe J, and Stäger S
   "The TLR7-IRF-5 axis sensitizes memory CD4+ T cells to Fas-mediated apoptosis during HIV-1 infection"

3. **Le colloque La Journée Québécoise du VIH – 2023 (National Academic Event, poster presentation), March 22, 2023. Montréal, Québec, Canada.**
   Carmona-Pérez L, Dagenais-Lussier X, van Grevenynghe J, and Stäger S
   "Upregulation of TLR7 and IRF5 sensitizes memory CD4 T cells from HIV1+ individuals on ART to cell death".

4. **9th Annual Montreal Immunology Meetings (MIM) symposium (National Academic Event, poster presentation), October 27, 2022. Montréal, Québec, Canada.**
   Carmona-Pérez L, Dagenais-Lussier X, van Grevenynghe J, and Stäger S.
   "Upregulation of TLR7 and IRF5 sensitizes CD4+T cells from HAART patients to cell death"

5. **Canadian Society for Immunology (CSI), 34th Annual Spring Meeting (National Academic Event, poster presentation), June 17 to 20, 2022. Halifax Québec, Canada.**
   Carmona-Pérez L, Dagenais-Lussier X, van Grevenynghe J, and Stäger S.
   "Upregulation of TLR7 and IRF5 sensitizes CD4+T cells from HAART patients to cell death"

6. **Le Congrès Armand-Frappier (National Academic Event, oral presentation) Online event, November 8 to 12, 2021. Montréal, Québec, Canada.**
   Carmona-Pérez L, Dagenais-Lussier X, van Grevenynghe J, and Stäger S.
   "The role of IRF-5 in CD4+ T cells in HIV+ individuals"

7. **8th Annual Montreal Immunology Meetings (MIM) symposium (National Academic Event, poster presentation) Online event, November 1, 2021. Montréal, Québec, Canada.**
   Carmona-Pérez L, Dagenais-Lussier X, van Grevenynghe J, and Stäger S
   "The role of IRF-5 in CD4+ T cell maintenance and survival in HIV+ individuals"

8. **Montreal Immunology Meetings (MIM) symposium (poster presentation), November 6, 2019. Montréal, Québec, Canada.**
   Carmona-Pérez L, Dagenais-Lussier X, van Grevenynghe J, and Stäger S.
   "The role of IRF-5 in CD4+ T cells during immune response to HIV"

9. **Le Congrès Armand-Frappier (poster presentation), Octobre 28 to 31, 2019. Saint-Sauveur, Québec, Canada.**
   Carmona-Pérez L, Dagenais-Lussier X, van Grevenynghe J, and Stäger S. Saint-Sauveur, Québec, Canada.
   "The role of IRF-5 in CD4+ T cells during immune response to HIV"