## Title
Defining Feline Adipose-Derived Mesenchymal Stem Cells-Induced Alterations on T-lymphocytes and Implications for the Treatment of Viral and Inflammatory Conditions



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# Defining Feline Adipose-Derived Mesenchymal Stem Cells-Induced Alterations on T-lymphocytes and Implications for the Treatment of Viral and Inflammatory Conditions

By

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# Defining Feline Adipose-Derived Mesenchymal Stem Cells-Induced Alterations on T-lymphocytes and Implications for the Treatment of Viral and Inflammatory Conditions


## Abstract

Feline adipose-derived mesenchymal stem cells (ASCs) engage with a variety of immune cells and have been used in clinical trials for the treatment of inflammatory and immune-dysregulated diseases with notable success in feline chronic gingivostomatitis (FCGS). FCGS is a chronic, inflammatory oral disease, secondary to hyperactive immune response with strong causal association with feline calcivirus (FCV). However, the mechanisms by which feline ASCs modulate T-cells and their implementation in virus-associated conditions have been less explored. We showed that feline ASCs limit T-cell proliferation by causing G0-G1 cell cycle arrest, using the soluble mediator prostaglandin E2 (PGE2) and I-CAM 1/LFA-1 ligand interaction. Additionally, Feline ASCs also shift CD8+ T-cells' phenotype toward terminally differentiated effector cells (CD57+, CD45R+, CD62L-) and upregulated granzyme B, IL-2 and KLRG-1 expression, enhancing their cytotoxic potential. Moreover, FCGS patients post-ASC therapy, had decreased level of T-cell activation in circulation and a lowered central memory CD8+ T-cell ($T_{CM}$) in lymphoid tissue, possibly indicative of resolved chronic antiviral response. Feline ASCs could aid in disease cure caused by an underlying persistent viral infection.

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# Multipotent Stromal Cells and Viral Interaction: Current Implications for Therapy

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**Abstract**
Multipotent stromal cells (MSCs) are widely utilized in therapy for their immunomodulatory properties, but their usage in infectious viral diseases is less explored. This review aimed to collate the current novel use of MSCs in virus-associated conditions, including MSC's susceptibility to virus infection, antiviral properties of MSCs and their effects on cell-based immune response and implementation of MSC therapy in animal models and human clinical trials of viral diseases. Recent discoveries shed lights on MSC's capability in suppressing viral replication and augmenting clearance through enhancement of antiviral immunity. MSC therapy may maintain a crucial balance between aiding pathogen clearance and suppressing hyperactive immune response.

**Keywords** Multipotent stromal cells · Mesenchymal stem cell · Virus interaction · Viral disease · Antiviral immunity

## Introduction

Multipotent stromal cells (MSCs) can be isolated from a variety of tissues, expanded ex-vivo, and administered to patients as a therapeutic agent. MSCs undergo differentiation into mesenchymal tissue types and secrete trophic factors to aid in the regeneration and repair of damaged tissue [1, 2]. MSCs further interact with various types of immune cells and their potent immunomodulatory properties are being investigated in numerous clinical trials [3].

MSCs inhibit NK cell and T-cell proliferation [4, 5], induce Treg differentiation [6], reduce the differentiation of B-cells to antibody-secreting plasma cells [7], and shift monocytes and dendritic cells to a regulatory phenotype [8, 9]. However, MSCs may exert differential effects depending on the local microenvironment [10], adding even more to the complexity of understanding MSC-mediated immunomodulation. MSC express pattern recognition receptors (PRR) such as Toll-like receptors (TLR), retinoic acid-inducible gene I-like receptors (RLRs) and nucleotide binding domain-like receptors (NLRs). Ligation of PRRs leads to downstream MSC cell signaling cascades and cell activation [11].

While MSC's immunomodulatory effects with a shift towards peripheral tolerance are very well studied, [12, 13] MSCs are also capable of augmenting anti-bacterial responses and can produce antimicrobial peptides, enhanced by the presence of bacteria [11, 14, 15]. While MSCs contribute to host defense and inflammation, there are limited data on MSC use in infectious diseases, particularly in viral infections.

The objectives of this review are 1) to collate current data on virus-MSC interactions and MSC interaction with cells that are at the forefront of anti-viral immunity, 2) to discuss the current status of MSC therapy in the context of viral diseases, including COVID-19, 3) to review animal models of viral disease and on-going human MSC clinical trials, and 4) highlight key gaps in knowledge and future research opportunities.

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## Viral Infection of MSCs, Potential Outcomes and Safety

MSCs are susceptible to infection by a wide variety of RNA and DNA viruses both in vitro and in vivo [16–23]. MSCs possess numerous functional surface receptors [24], which potentially could facilitate viral entry. Although virus receptors vary in structure and function, they are more inclined to utilize molecules involved in cellular adhesion [25], for example, I-CAM1 which MSCs express and use for transmigration and immunomodulation [26].

MSC surface receptor expression and viral tropism may partially explain MSC susceptibility to viral infection. MSCs are highly permissive to infection by many genera of Herpesviruses, including Herpes Simplex-1 (HSV-1), Varicella Zoster virus (VZV) and Cytomegalovirus (CMV) [27]. HSV-1 can infect MSCs through the heparan sulfate receptor [28]. However, Epstein-Barr virus (EBV) and Human Herpesvirus-6, 7 and 8 (HHV) were unable to infect human MSCs despite MSC expression of receptors known to facilitate viral entry into other cells [29, 30]. This may be explained by cellular tropism as EBV typically resides in B-cells and HHV typically infects T-cells while HSV-1, VZV and CMV primarily infect epithelial cells at various sites of mucosal membranes [31].

Various factors play a role to determine viral tropism, including surface binding receptors on target cells, antiviral signaling of cytokines, availability of intracellular host factors which supports viral RNA/DNA synthesis and activation state of the cell [32].

Although MSCs clearly are susceptible to viral infection, MSCs possess some resistance not observed in other somatic cells, partially due to their intrinsic upregulation of interferon-stimulated genes (ISGs) [33]. Stem cell pluripotency has been correlated to their resistance to viral infection and the resistance to viral infection is more robust in embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) and less so in highly differentiated cells, as shown an in vitro study where MSC-derived cells were permissive to HIV-1 infection, but that same virus could not productively infect undifferentiated MSCs [34].

Outcomes of viral entry into MSCs are variable and may result in either MSC death, persistent infection or cellular transformation which impairs their functionality. In general, RNA viruses are more effective in initiating pro-inflammatory cytokine production and eliciting an immediate antiviral response from MSCs (Fig. 1 and Table 1). This is in line with the strategy that infection with the majority of RNA viruses, with the exception of retroviruses, is more likely to cause an acute infection rather than chronic conditions, compared to DNA viruses [35]. Additionally, RNA viruses have more disordered viral protein packaging and a small genome size. These factors may advantage the prompt initiation of conformational changes necessary during host-cell entry and interaction [36]. For example, Chikungunya virus (CHIKV) can dampen MSC's osteogenic differentiation, potentially limiting their use in regenerative medicine [37]. However, the ability to elicit an inflammatory reaction is not solely limited to RNA viruses. Parvovirus B19, a single-stranded DNA virus,

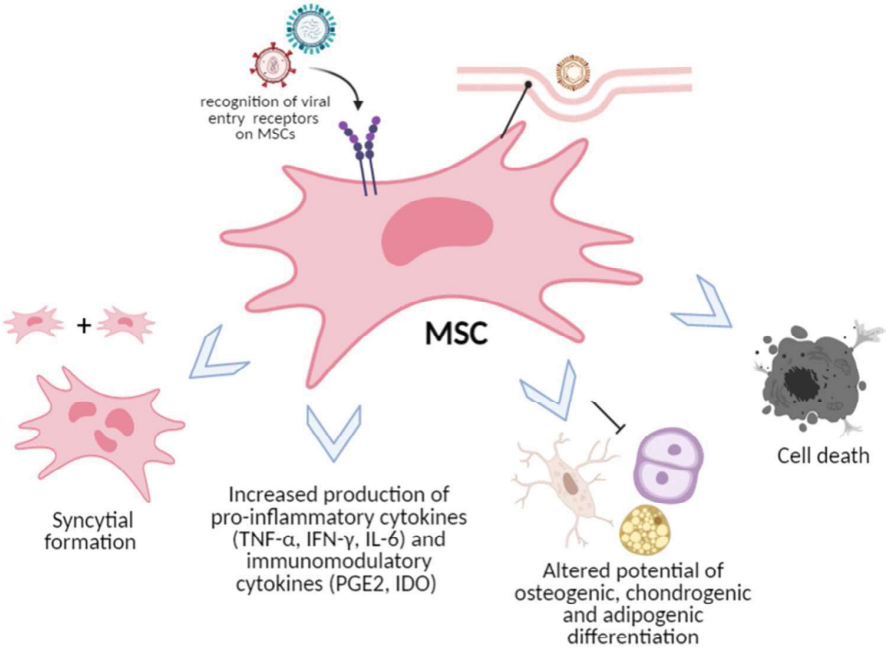


**Fig. 1** Possible outcomes of viral infection on mesenchymal stem cells. TNF-α: tumor necrosis factor-alpha; IFN-γ: interferon-gamma; IL-6: interleukin-6; PGE2: prostaglandin E2; IDO: indoleamine-2,3-dioxygenase

**Table 1** Viral susceptibility of MSCs

| Family | Virus | Type | MSC sources and species | Outcome of Infection |
|---|---|---|---|---|
| Pneumoviridae | Respiratory syncytial virus (RSV) [19] | ssRNA | Human BM-MSCs | Increased expression of IFN-β and IDO, enhanced MSC's capability of PBMC inhibition of proliferation |
| Orthomyxoviridae | Human and Swine Influenza virus (H1N1) [21] | ssRNA | Human/Porcine BM-MSCs | Increased production of pro-inflammatory cytokines (TNF-α and IL-6), CPE |
| | Avian Influenza virus (H1N1 and H9N5) [38]<br>(H5N1) | | Chicken pulmonary MSCs<br>Human UC-MSCs and BM-MSCs | Increased production of cytokines (IL-6 and IL-8), CPE |
| Retroviridae | Human Immunodeficiency Virus (HIV) [39, 40]<br>Feline Foamy Virus (FFV) [18]<br>Simian Foamy Virus (SFV) [41] | ssRNA | Human BM-MSCs<br>Feline AD-MSCs<br>Rhesus Monkey BM-MSCs | Increased adipogenic potential, Impaired osteogenic differentiation, Induced senescence<br>Syncytial formation, Impaired proliferation, CPE |
| Togaviridae | Chikungunya virus (CHIKV) [37] | ssRNA | Human BM-MSCs | Impaired osteogenic differentiation |
| Birnaviridae | Infectious bursal disease virus (IBDV) [42] | dsRNA | Chicken BM-MSCs | CPE |
| Herpesviridae | Cytomegalovirus (CMV), Herpes simplex virus type 1 (HSV-1) [29]<br>Varicella zoster virus (VZV), HSV6-8, [16]<br>Kaposi sarcoma-associated herpesvirus [43] | dsDNA | Human BM-MSCs, Human fetal MSCs | CPE<br>Impaired immunosuppressive function (CMV) |
| Hepadnaviridae | Hepatitis B virus (HBV) [22] | dsDNA | Human BM-MSCs | Maintained MSCs characteristics |
| Parvoviridae | Parvovirus B19 virus [17, 44] | ssDNA | Human synovial and BM-MSCs | Maintained MSCs characteristics [44]<br>Increased expression of IL-6 and TNF-α [17] |

CPE: cytopathic effects (MSC lysis), BM-MSCs: bone marrow derived MSCs,
UC-MSCs: umbilical cord derived MSCs

can infect human BM-MSCs with resultant upregulation of pro-inflammatory cytokine gene expression, such as, IL-6 and TNF-α [17].

To illustrate the consequences of DNA virus infection, MSCs infected with CMV lost their cytokine-induced immunomodulatory function and were no longer capable of inhibiting microbial growth [23]. In addition, the US11 protein utilized by CMV for immune evasion can also downregulate MHC class I expression on human MSCs, making them vulnerable to NK cell-mediated lysis [45]. This same effect was described in horse MSCs after equid herpesvirus-1 (EHV-1) infection [20].

The ability of viruses to enter and alter host MSCs may also be host species dependent. Human and murine MSCs secrete different immunomodulatory mediators [46] and the efficacy of these mediators to limit or enhance viral replication may be an important determinant of infection outcome. For example, indoleamine-2,3-dioxygenase (IDO) is a primary mediator utilized to mitigate viral replication in human MSCs, but the same effect was not observed in murine MSCs [47].

Due to the concern over the ability of viruses to infect MSCs, tissues from candidate donors are screened for common viral infection and expanded allogeneic MSC doses are also screened for the presence of viral infection prior to cell administration. Although allogeneic MSCs may potentially serve as a reservoir for latent viruses, especially if administered to immunocompromised recipients, clinical studies in GvHD patients have shown that MSC treatment did not induce more viral reactivation as compared with conventional immunosuppressive therapy [48]. Overall, current data suggests that MSC therapy is deemed largely safe for with minimal virus-associated risk.

## Anti-Viral Properties of MSCs

Despite their permissiveness to some viral entry, evidence has also emerged that MSCs can mitigate viral infection via upregulation of their antiviral mechanisms. MSCs are more resistant to viral infections when compared to more differentiated cells through their intrinsic upregulation of IFN-stimulated genes (ISG) which block viral replication and propagation [33]. Moreover, silencing ISG such as p21/CDKN1A and IFITM3 expression in MSCs, resulted in increased susceptibility of MSCs to chikungunya virus infection and zika virus respectively [33, 49].

Several in vitro studies have demonstrated antiviral activity of MSCs, for example, MSCs can inhibit inflammasome activation in the presence of Coxsackievirus B3 [50] Another described antiviral mechanism of MSCs is through their non-coding miRNAs. Some MSC's released miRNAs demonstrated vigorous antiviral activity that could inhibit Hepatitis C virus infection [51]. With these limited data, primary mechanism of extracellular viral inhibition may possible be from secreted trophic factors.

In vivo murine studies of influenza virus-induced acute lung injury have demonstrated that MSC administration reduced pulmonary injury and inflammation and restored alveolar fluid clearance [50, 52]. In a mouse model of murine gammaherpesvirus-68 (MHV-68) infection, MSCs also showed anti-herpesviral properties, mediated by a cytosolic DNA sensing pathway, and MSCs also limited intracellular viral replication in IFN-γ dependent and independent manners [53]. However, the ability of MSCs to inhibit viral replication in vivo and the mechanisms involved require further investigation.

## Effects of MSCs on the Cell-Based Immune Response Against Viral Infection

Aside from their intrinsic restriction factors, MSCs can also modify the antiviral response of the immune cells normally implicated in antiviral defenses. MSCs can interact with and influence both the innate and adaptive immune cellular components, primarily on NK cells and T-cells, potentially altering the outcome of the response to viral infection (Fig. 2).

Despite their intensive studies, the data on MSC interactions with NK and CD8 + T-cells are conflicting and complex. Autologous and allogeneic cultured MSCs can be recognized and killed by activated NK cells. However, IFN-γ primed MSCs, mimicking exposure to inflammatory environment, upregulate MHC class I expression and avoid NK-cell mediated destruction [54]. While they can be targets, MSCs can also influence and alter NK cells' phenotype. Human MSCs inhibited NK cell proliferation, decreased cytokine production and dampened cell differentiation to fully functional effector cells in vitro. These effects were mediated by the soluble mediators IDO and prostaglandin E2 (PGE2) and resulted in the downregulation of NK cell surface receptors [55]. A more recent study demonstrated that MSCs upregulated NK cell secretion of IFN-γ and TNF-α and also triggered their degranulation, increasing the release of perforin and granzyme and enhancing NK cell's effector phenotype [56]. The discrepancies may be due to variations in study designs. The first study examined pre-activated human NK cells that had been cultured with IL-2 for 7 days while the latter study used short-term activated human NK cells cultured with a more diverse combination of cytokines. Furthermore, the ratios of NK cells to MSCs in experimental settings also play a role in demonstrated level of suppression [57].

With CD8 + T-cells, it is well established that MSCs can inhibit T-cell proliferation [58, 59] through the release

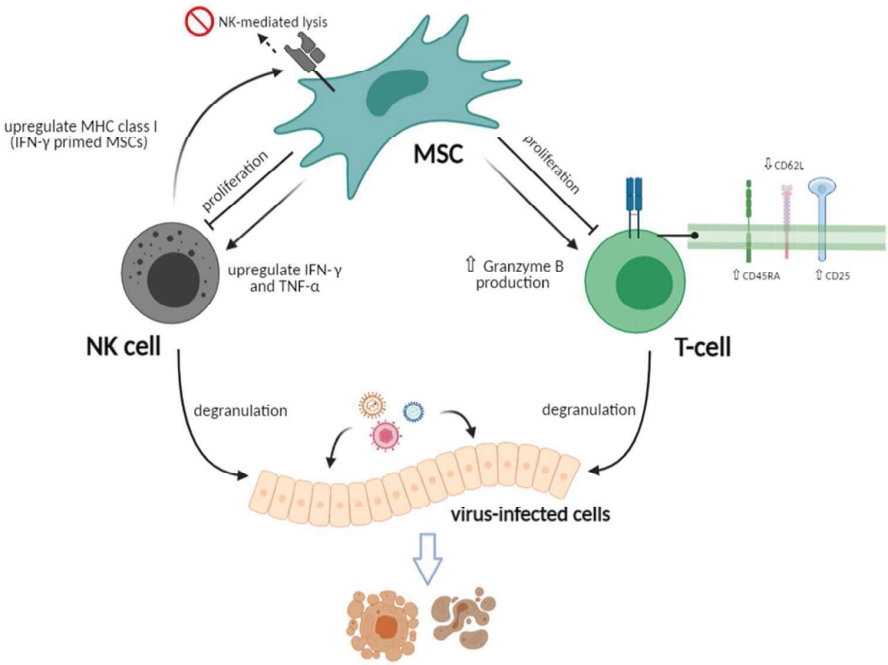


**Fig. 2** MSCs-induced alterations on NK cells and T-cells. TNF-α: tumor necrosis factor-alpha; IFN-γ: interferon-gamma

of transforming growth factor beta (TGF-β) and hepatocyte growth factor (HGF), which leads to the decrease of cyclin D2, causing proliferation arrest in the G0G-1 phase of cell cycle [60]. However, MSCs do not appear to hinder CD8 + T-cell cytotoxicity function. After exposure to an exogenous peptide, CD8 + T cells retained the ability to lyse target cells even in the presence of human MSCs [61]. Murine MSCs enhanced granzyme B production and induced degranulation of activated CD8 + T-cells in vitro [62] yet this upregulating effect was not demonstrated in human MSCs [63]. MSCs may reduce cytotoxicity-mediated lysis, only when the T-cells had not been pre-activated (naïve) [64] which may not emulate realistic clinical conditions where MSC therapy would be administered and expected to exert their effects on antigen-experienced CD8 + T-cells.

Additionally, MSCs did not affect the expansion and function of virus-specific CD8 + T-cell in the context of EBV and CMV infection. However MSCs were capable of suppressing alloreactive T-cells [65]. A separate study showed that MSCs may inhibit proliferation of virus-specific CD8 + T-cells, but the experiment was performed by briefly pulsing T-cells with CMV phosphoprotein and Influenza matrix protein antigen for 2 h [66]. This activation may result in a less robust outcome compared to the generation of virus-specific T-cells for 14 days to mimic actual viral exposure. IFN-γ derived from MSCs was hypothesized to play a role in offsetting the immunosuppressive effect of MSCs by mediating the partial cytotoxic responses during viral infection [67].

In addition to affecting T-cell proliferation, MSCs can also alter the activation and differentiation process of T cells. MSCs are known to promote generation of regulatory T-cells [68]. A recent study shed light that this promotion of regulatory T-cells from MSCs arises from an epigenetic conversion of conventional T cells to regulatory phenotype rather than expansion of natural regulatory T-cells [69], increased population of regulatory T-cells have been shown to improve influenza virus clearance in a murine study [70].

The ability of MSCs to alter immune cell functions is likely dependent on host species and varying inflammatory conditions. Several contradictory findings showed that MSCs were unable to suppress or even enhance T cell responses under several conditions. Since MSCs respond differentially to the dynamic changes of inflammatory factors, the immunoregulation of MSCs is distinctly plastic [10] and does not occur intrinsically, but activated by certain combinations of inflammatory cytokines, IFN-γ with TNFα, or IL-1β [71]. Therefore, MSCs' impact on subsets of virus-associated immune cells will differ under various pathological settings.

## MSC Therapy in Animal Models of Viral Diseases

MSCs and/or their secretome have been used as a therapy in several animal models of viral diseases. These studies have demonstrated that MSCs can reduce inflammation and dampen pro-inflammatory cytokine production, but also demonstrated more novel outcomes including targeting viral-sequestered cells and tissues and directly inhibiting viral expression and replication (Table 2).

The murine model has been used to study Japanese encephalitis virus (JEV), Hepatitis B and Coxsackievirus B3 virus (CVB). Bone marrow (BM)-derived MSCs have been used to treat all 3 viral infections in the mouse model. JEV is the leading cause of viral encephalitis in Asia and the mouse has disease manifestations that mimic the symptoms and biomarkers observed in humans [72]. The administration of intravenous murine BM-MSCs to mice infected with JEV resulted in a direct antiviral effect both in vitro and in vivo as evidenced by a reduction of viral load in cerebral tissue, decreased inflammatory response and neuronal damage and a reduction of viral propagation in Neuro2a cells in co-culture with MSCs [73]. This antiviral effect of murine BM-MSCs was found to be mediated by the induction of IFN-α and β expression in infected cells. The JEV study by Bian et. al is one of the first animal studies to demonstrate a novel concept that MSCs can directly hinder viral replication in vivo.

CVB infection in the mouse results in myocarditis initiated both by immune-mediated mechanisms and by direct viral-induced cardiomyocyte injury. Similar to JEV, the administration of MSCs reduced intracellular viral particle production and viral progeny release in cardiomyocytes, and dampened CVB-induced excessive T-cell proliferation that results in myocardial injury in a nitric-oxide (NO) dependent manner [74].

In a mouse model of acute HBV infection [75], the adoptive transfer of BM-MSCs ameliorated liver injury and decreased inflammation. However, the therapy also paradoxically increased viral replication, hypothesized to be partly due to MSC suppression of NK-mediated cell cytotoxicity. NK cells play a crucial role in viral clearance during acute HBV infection. This study did not explore MSCs' effect on CD8 + T-cell function and focused mainly on short-term outcome without observing long-term progression of HBV infection post-MSC therapy.

In additional to cell-based approach, MSC-derived extracellular vesicles (EVs) may have comparable efficacy to MSC administration in a swine model of influenza. Khatri et. al found that the systemic administration of EVs isolated from swine BM-MSCs reduced nasal virus shedding and viral replication in lung tissue. The administration of

**Table 2** Multipotent stromal cells and their exosome-derived components in the treatment of viral diseases

| Animal Models | | | | |
|---|---|---|---|---|
| Target Virus | Treatment | Species | Viral Kinetics | Outcome |
| Japanese Encephalitis Virus (JEV) | BM-MSCs | Mice | Decreased viral load in brain tissue and reduced viral propagation | Reduced mortality, alleviated inflammatory response (neuronal damage and blood brain barrier destruction) |
| Coxsackievirus B3 (CVB3) | BM-MSCs | Mice | Reduced intracellular viral particle production and viral progeny release in cardiomyocytes | Improved CVB3-induced myocarditis, mitigated cardiac apoptosis, cardiomyocyte damage and cardiac mononuclear cell activity |
| Hepatitis B Virus | BM-MSCs | Mice | Enhanced viral gene expression and replication in vivo | Attenuated immune-mediated liver injury, reduced pro-inflammatory cytokine production cellular response |
| Swine Influenza Virus | MSCs derived exosomal microRNAs | Pig | Inhibited viral replication lung tissue and reduced viral shedding in nasal swabs | Alleviated SwIV-induced acute lung injury and decreased production of pro-inflammatory cytokines |
| Simian Immunodeficiency Virus | Allogeneic BM-MSC | Rhesus macaque | Decreased peripheral viral loads | Clearance of virus from gut effector sites, robust regeneration of germinal centers and restoration of follicular T helper cells |
| *In vitro* experiments | | | | |
| Hepatitis C Virus | Umbilical MSCs derived exosomes | Human | Inhibition of viral infection through paracrine signaling with synergistic effect upon combination with IFN. No effects on HCV entry into target cells | |
| Human Immunodeficiency Virus (HIV) | AD-MSCs | Human | Enable potent HIV reactivation in latently infected monocytic and T-cell lines through PI3K-NFκB signaling pathway | |

EVs also altered pro-inflammatory mediator secretion and reduced histopathological evidence of injury when administered after viral inoculation in a mixed swine (H3N2, H1N1) and avian (H9N5, H7N2) influenza-induced pig lung injury model. The authors hypothesized that there was RNA transfer from EVs to epithelial cells [76]. These findings suggest systemic EV administration as a potential cell-free strategy for use in respiratory virus-induced lung conditions.

In our recent unpublished work, we have determined that feline adipose-derived MSCs were able to enhance granzyme B expression in CD8 + T-cells, shift their phenotype towards terminally differentiated effector cells (CD57 +, CD45RA + and CD62L-) and augment the ability of these cells to lyse virally-infected target cells in vitro [article under review]. Preliminary in vivo data also suggest that in feline chronic gingivostomatitis (FCGS), a disease associated with feline calicivirus infection, a positive response to MSC therapy also results in FCV clearance aligned with improvement in clinical disease [77].

Similarly, in an unpublished study of SIV infection model of AIDS in rhesus macaques, our group has demonstrated that MSCs enhance viral clearance by augmenting CD8 + T-cell and B cell activity through granzyme B upregulation and increased anti-SIV antibody production, respectively. In this model, MSCs enhanced viral particle transport to intestinal lymphoid follicles, and the development of robust germinal centers via Tfh induction which lead to enhanced viral clearance. Moreover, MSC treatment of SIV + rhesus macaques induced proliferation of CD8 + T-cells, in contrary to the suppression normally observed with MSC therapy [article under review].

Collectively, these studies in animal models suggest that MSCs have the ability to adapt their interactions with immune cell subsets in viral diseases in ways that are distinct from MSC interaction with immune cells when administered for diseases driven by immune-pathology mechanisms which aim to enhance the regulatory arms of the immune system.

## MSC Therapy in Human Clinical Trials of Viral Diseases

Upper until the time of article submission, there are currently 19 clinical studies registered involving the use of MSCs and/or their secretome to treat viral infection or the conditions associated with viral infection (ClinicalTrials.gov, Table 3). The most common therapeutic target (12/19; 63.2%) was for the treatment of respiratory problems associated with the novel Coronavirus infection (SARS-CoV-2; Covid-19). These trials are predominantly conducted with umbilical-cord derived MSCs (UC-MSCs). UC-MSCs are desirable for the treatment of acute viral infections due to their rapid doubling time in culture compared to BM-MSCs or AD-MSCs [78]. Robust MSC expansion facilitates the rapid generation of a therapeutic MSC dose in critically ill patients [79].

Clinical trials using MSC therapy for Hepatitis B virus (HBV) infection and its associated liver disease are also reported (4/19; 21.1%). HBV infection is extremely widespread with over 350 million carriers around the world. HBV infection can result in hepatitis cirrhosis and hepatocellular carcinoma and there are limited treatment options available [80]. In a randomized controlled trial for HBV–related acute-on-chronic liver failure, infusion of allogeneic BM-MSCs significantly increased survival rate by improving liver function and decreasing the incidence of severe concurrent infections [81]. Based on a meta-analysis of MSC-based clinical trials for liver diseases, mechanisms involved in the efficacy of MSC therapy in HBV are focused on the hepatic reparative effects and/or restoration of T-reg/Th17 balance rather than on viral clearance [82]. With that said, concurrent in vitro work showed that BM-MSCs inhibited the expression of HBV DNA and enhanced viral clearance in HBV-infected lymphocytes [83]. Since BM-MSCs permit HBV infection, they may become reservoir of viruses after administration. However, AD-MSCs were found to be not susceptible to HBV [84] and may be a more suitable source of HBV-associated liver condition. Further studies on the long-term effects of MSC therapy on HBV infected individuals, from varying sources of MSCs, are necessary to determine its safety and efficacy in therapeutic use.

Clinical trials have also been conducted in patients with CMV and human immunodeficiency virus (HIV) infection. CMV is common, but infection is often asymptomatic except for in immunocompromised patients. CMV also remains a common complication after hematopoietic stem cell transplantation [85]. Paradoxically, CMV can infect and compromise MSC functions [86]. MSC therapy in the context of CMV may not be truly efficient and should be reserve for refractory cases as a further down option.

MSC therapy is also being applied to HIV infection as an adjunct for immunomodulation. For many patients, highly effective anti-retroviral therapy (HAART) can suppress circulating viral load and can increase life expectancy. However, some infected individuals are classified as nonimmune responders (NIR) and remain susceptible to opportunistic infections due to their low numbers of CD4 + T-cells. An in vitro study using latent HIV-infected cell lines reported a novel role for MSCs and MSC secretome in HIV-1 latency-reactivation through phosphoinositide 3-kinase (PI3K) and nuclear factor kappa-B (NFκB) signaling pathways [87]. A clinical trial published by Zhang et. al showed that UC-MSC therapy can increase the number of circulating naive and central memory CD4 + T-cells and restore HIV-specific IFN-γ and IL-2 production, evidence of systemic immune reactivation

**Table 3** Current clinical trials on the treatment of viral-associated diseases with multipotent stromal cells and/or their products

| | Study Title | Treatment | Conditions | Status | Location |
|---|---|---|---|---|---|
| 1 | Treatment with Human Umbilical Cord-derived Multipotent stromal cells for Severe Corona Virus Disease 2019 (COVID-19) | UC-MSCs | Corona Virus Disease 2019 (COVID-19) | Completed | China |
| 2 | Efficacy and Safety of Umbilical Cord Multipotent stromal cells for the Treatment of Severe Viral Pneumonia | UC-MSCs | Corona Virus Disease 2019 (COVID-19) | Not yet recruiting | China |
| 3 | Umbilical Cord(UC)-Derived Multipotent stromal cells(MSCs) Treatment for the 2019-novel Coronavirus (nCOV) Pneumonia | UC MSCs | Corona Virus Disease 2019 (COVID-19) | Recruiting | China |
| 4 | Study of Human Umbilical Cord Multipotent stromal cells in the Treatment of Severe COVID-19 | UC-MSCs | Corona Virus Disease 2019 (COVID-19) | Not yet recruiting | China |
| 5 | Bone Marrow-Derived Mesenchymal Stem Cell Treatment for Severe Patients With Coronavirus Disease 2019 (COVID-19) | BM-MSCs | Corona Virus Disease 2019 (COVID-19) | Not yet recruiting | China |
| 6 | A Pilot Clinical Study on Inhalation of Multipotent stromal cells Exosomes Treating Severe Novel Coronavirus Pneumonia | MSC-derived exosomes | Corona Virus Disease 2019 (COVID-19) | Not yet recruiting | China |
| 7 | Use of UC-MSCs for COVID-19 Patients | UC-MSCs | Corona Virus Disease 2019 (COVID-19) | Completed | USA |
| 8 | Umbilical Cord Tissue (UC) Derived Mesenchymal Stem Cells (MSCs) Versus Placebo to Treat Acute Pulmonary Inflammation Due to COVID-19 | UC-MSCs | Corona Virus Disease 2019 (COVID-19) | Not yet recruiting | USA |
| 9 | Regenerative Medicine for COVID-19 and Flu-Elicited ARDS Using Longeveron Mesenchymal Stem Cells (LMSCs) | MSCs (unspecified source) | Corona Virus Disease 2019 (COVID-19) | Recruiting | USA |
| 10 | Clinical Use of Stem Cells for the Treatment of Covid-19 | MSCs (unspecified source) | Corona Virus Disease 2019 (COVID-19) | Recruiting | Turkey |
| 11 | Treatment of Covid-19 Associated Pneumonia with Allogenic Pooled Olfactory Mucosa-derived Multipotent stromal cells | OM-MSCs | Corona Virus Disease 2019 (COVID-19) | Enrolling by invitation | Belarus |
| 12 | Therapeutic Study to Evaluate the Safety and Efficacy of DW-MSC in COVID-19 Patients | MSCs (unspecified source) | Corona Virus Disease 2019 (COVID-19) | Completed | Indonesia |
| 13 | Therapeutic Effects of Liver Failure Patients Caused by Chronic Hepatitis B After Autologous MSCs Transplantation | BM-MSCs | Liver Failure from HBV infection | Completed | China |
| 14 | Allogeneic Bone Marrow Multipotent stromal cells Transplantation in Patients with Liver Failure Caused by Hepatitis B Virus (HBV) | BM-MSCs | Liver Failure from HBV infection | Unknown | China |
| 15 | Clinical Study of Human Umbilical Cord Multipotent stromal cells(19#iSCLife®-LC) in the Treatment of Decompensated Hepatitis B Cirrhosis | UC-MSCs | Liver Cirrhosis from HBV infection | Recruiting | China |

**Table 3** (continued)

| | Study Title | Treatment | Conditions | Status | Location |
|---|---|---|---|---|---|
| 16 | Umbilical Cord Mesenchymal Stem Cell for Liver Cirrhosis Patient Caused by Hepatitis B | UC-MSCs | Liver Cirrhosis from HBV infection | Recruiting | Indonesia |
| 17 | MSC for Treatment of CMV Infection | MSCs (unspecified source) | Cytomegalovirus infection after allogeneic hematopoietic stem cell transplantation | Unknown | China |
| 18 | Treatment With MSC in HIV-infected Patients With Controlled Viremia and Immunological Discordant Response | AD-MSCs | HIV | Completed | Spain |
| 19 | Umbilical Cord Mesenchymal Stem Cells for Immune Reconstitution in HIV-infected Patients | UC-MSCs | HIV | Unknown | China |

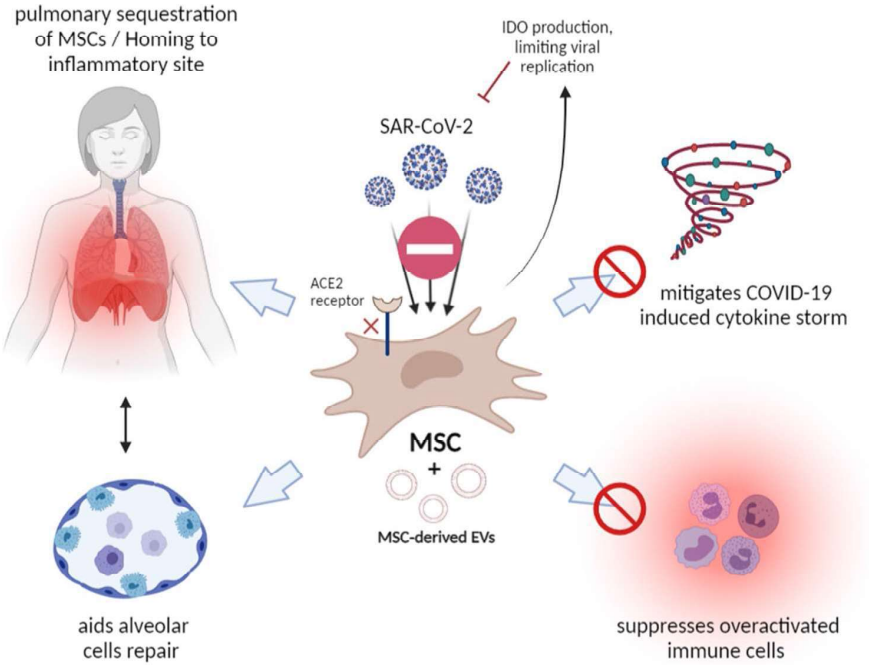


**Fig. 3** Potential use of MSC for the treatment of COVID-19

post-treatment and did not lead to increased viral loads [88]. However, in a early phase clinical trial for NIR, MSC infusions were found to not effectly improve immune recovery or reduce immune overactivation [89]. Supplemental in vivo research is needed to elucidate the effects of MSCs in reactivation of HIV-1 in host microenvironment.

## MSCs for the Treatment of COVID-19

COVID-19, a newly-recognized infectious disease with rapid transmission of severe acute respiratory syndrome coronavirus 2 (SAR-SCoV-2) and has become a major concern all over the world. During the rush of finding novel treatment for the COVID-19 pandemic in addition to the traditional corsticosteroid therapy, covalescent plasma and neutralizing antibody cocktails [90–92], MSCs and their secreted products were explored as viable options due to their antiviral, anti-inflammatory and tissue regenerative capabilities (Fig. 3).

MSCs have been known to be sequestered in the lung after intravenous administration, creating a benefit in their utilization for the treatment of pulmonary disease [93]. Moreover, MSC-derived extracellular vesicles have shown to ameliorate inflammatory lung diease, including respiratory distress syndrome (ARDS), acute lung injury (ALI) and chronic

obstructive pulmonary disease (COPD), from both infectous and non-infectious causes in several preclinical models [94]. In a completed clinical trial involving acute respiratory distress syndrome (ARDS) induced by epidemic influenza A (H7N9), MSC therapy significantly improved patients' survival rate, lung function and decreased lung fibrosis [95].

Recent in vitro work [96] showed that despite their expression of angiotensin converting enzyme 2 (ACE2), a receptor for SAR-CoV-2 entry, human MSCs were resistant to their infection under steady-state, inflammatory condition and in the presence of SAR-CoV-2 infected cells. Moreover, SAR-CoV-2-exposed MSCs also retained their ability to secrete IDO [96], a mediator that can limit emergent viral biosynthesis through tryptorphan depletion pathway [97].

MSC therapy was implemented toward SAR-CoV-2 infection-induced pneumonia for the first time by Leng et. al in January, 2020 in 7 Covid-19 patients with promising outcomes. MSC administration was associated with an increase in the peripheral blood lymphocyte count and a concurrent decrease in C-reactive protein and activated cytokine-secreting immune cells, such as CXCR3+CD4+/CD8+T cells and CXCR3+NK cells [98]. Additionally, these Covid-19 patients became SAR-CoV-2 virus negative through RT-PCR detection 2 weeks after MSC administration [98]. According to the first published study, MSCs cannot be infected by SARS-CoV-2 and are deemed safe and effective in critical patients. The limitation of the aforementioned first study includes the lack of sufficient control group, small sample size and enrolled participants with only 1 patient in critical condition. However, several recent clinical trials and case reports reaffirm the beneficial use of MSCs in COVID-19.

In a few recent double-blinded, phase-2 randomized control trials utilizing UC-MSCs for acute respiratory distress syndrome, MSCs therapy significantly improved survival, reduced inflammatory cytokines and alleviated COVID-19 induced lung damage [99, 100]. The clinical trial conducted by Langzoni et. al also measured mean viral load of SAR-CoV-2 through qRT-PCR on day 0 and day 6 of treatment which did not differ between treatment and control group. However, it is interesting to note that some of the patients in their control group remained SAR-CoV-2 positive while all of the participants in group receiving UC-MSCs were negative [99]. Further kinetic study of viral clearance may be befinicial, given that viral load of SAR-CoV-2 has been shown marked correlation to the severity of acute respiratory distress syndrome [101].

Another trial using UC-MSCs and placental MSCs showed that COVID-19 patients with low white cell count and low lymphocyte count prior to therapy resulted in poorer outcomes [102], suggesting that aside from reduction of inflammatory responses through secreted paracrine factors, MSCs' interaction with existing immune cells play an important role in successful treatment.

Overall, present data demonstrated in short-term studies that MSCs have shown efficacy in managing COVID-19 patient conditions with no obvious adverse effects. Pre-existing condition and other co-morbidities which may affect the potency of MSC therapy requires further investigation and confirmation.

## Conclusion

MSC therapy remains an option for the treatment of virus-associated diseases, especially those with sustained inflammation or those that require immunomodulation of skewed immune cell subsets. However, with certain virus infections, MSC therapy may result in enhanced viral replication, particularly in those in which MSCs are highly permissive to infection. In these cases, a cell-free therapeutic approach using EVs-derived from MSCs might be a promising approach to circumvent this issue.

Through TLR signaling and crosstalk between MSCs and effector immune cells, MSCs may maintain a crucial balance between enhancement of pathogen clearance and suppression of an overactive response. This interaction may help to preserve host cell integrity and facilitate tissue repair.

MSCs may react differentially under varying viral-associated conditions. It's crucial to bridge the translational gap between the fundamental research of MSCs and their therapeutic applications. Studies that directly evaluate the ability of MSCs to clear virus are still limited. More robust animal models of viral diseases with larger sample size and well-designed randomized controlled clinical trials are needed to adequately assess their safety and potential of exerting antiviral effects on various viruses.

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**Code Availability** Not applicable

## Declarations

**Ethics Approval** Not applicable

**Consent to Participate** Not applicable

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# Mechanisms utilized by feline adipose-derived mesenchymal stem cells to inhibit T lymphocyte proliferation

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## Abstract

**Background:** Feline adipose-derived mesenchymal stem cells (ASCs) have been successfully used in clinical trials for the treatment of immune-mediated diseases with T cell dysregulation. However, the immunomodulatory pathways utilized by feline ASCs to suppress T cell activation have not been fully determined. We investigated the mechanisms used by feline ASCs to inhibit T cell proliferation, including the soluble factors and the cell-cell contact ligands responsible for ASC-T cell interaction.

**Methods:** The immunomodulatory activity of feline ASCs was evaluated via cell cycle analysis and in vitro mixed leukocyte reaction using specific immunomodulatory inhibitors. Cell-cell interactions were assessed with static adhesion assays, also with inhibitors.

**Results:** Feline ASCs decrease T cell proliferation by causing cell cycle arrest in G0–G1. Blocking prostaglandin ($PGE_2$), but not IDO, partially restored lymphocyte proliferation. Although PDL-1 and CD137L are both expressed on activated feline ASCs, only the interaction of intercellular adhesion molecule 1 (ICAM-1, CD54) with its ligand, lymphocyte function-associated antigen 1 (LFA-1, CD11a/CD18), was responsible for ASC-T cell adhesion. Blocking this interaction reduced cell-cell adhesion and mediator (IFN-γ) secretion and signaling.

**Conclusions:** Feline ASCs utilize $PGE_2$ and ICAM-1/LFA-1 ligand interaction to inhibit T cell proliferation with a resultant cell cycle arrest in G0–G1. These data further elucidate the mechanisms by which feline ASCs interact with T cells, help define appropriate T cell-mediated disease targets in cats that may be amenable to ASC therapy, and may also inform potential translational models for human diseases.

**Keywords:** Mesenchymal stem cell, Adipose tissue, Feline, Immunomodulation, soluble mediators, ligands

## Background

Mesenchymal stem cells (MSCs) are a heterogeneous, multipotent stromal cell population, capable of proliferating in vitro as plastic-adherent cells with fibroblast-like morphology and differentiating into bone, cartilage, and adipose cells [1]. Aside from their regenerative properties, MSCs also possess immunomodulatory properties and have been used extensively to treat a wide variety of immune-mediated diseases, both in human and veterinary medicine [2]. In veterinary medicine, adipose-derived mesenchymal stem cells (ASCs) have been successfully used to treat cats with feline chronic gingivostomatitis (FCGS), a severe debilitating oral immune-mediated disease [3, 4], but the mechanisms by which feline ASCs modulate the immune system have not been fully elucidated.

MSCs can be immunosuppressive and can inhibit the mitogen-induced response of naïve T lymphocytes [5], both CD4+ and CD8+, as well as of natural killer (NK) cells [6]. There are several possible mechanisms by which MSCs may inhibit T lymphocyte proliferation including the induction of apoptosis, cell cycle arrest, induction of a phenotype switch to regulatory T cells, or decreasing T lymphocyte activation, ultimately leading

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to anergy [7]. Suppression of T cell proliferation by MSCs can be mediated by secreted soluble factors because the separation of MSCs and activated T lymphocytes by a transwell can inhibit proliferation without the presence of cell-cell contact [8, 9]. However, direct cell-cell contact is also important in MSC regulation of T lymphocyte function in both humans and cats [8, 10].

MSC immunosuppressive functions require preliminary activation by immune cells through the secretion of IFN-γ, a pro-inflammatory cytokine [6, 11]. Activated feline ASCs secrete high levels of immunomodulatory mediators, including indoleamine 2,3 dioxygenase (IDO), prostaglandin E2 ($PGE_2$), interleukin (IL)-6, IL-8, and transforming growth factor beta (TGFβ) similar to human MSCs. However, unlike human MSCs, the secretion of $PGE_2$ and IDO by feline ASCs is significantly reduced in the absence of direct-cell contact [8]. In contrast to other species, including humans, dogs, and horses, feline ASCs inhibit lymphocyte proliferation in the context of significantly increased concentration of IFN-γ [8, 12, 13].

Several cell ligand pairs have been implicated in MSC and T lymphocyte adhesion and signaling that subsequently impact the secretion of soluble immunomodulatory factors. These ligand pairs include ICAM-1/LFA-1, VCAM-1/VLA-4, and PDL-1/PD-1 [14, 15]. In murine MSCs, ICAM-1 is a requirement for lymphocyte–MSC adhesion and blocking ICAM-1 ligand reduced T cell accumulation around MSCs and reversed the suppression of lymphocyte proliferation [15]. ICAM-1 also plays a crucial role, particularly in T cell interactions with antigen-presenting cells, and is essential for the immunosuppressive effects of murine bone marrow-derived MSCs [15, 16]. PDL1 and PDL-1, a negative costimulatory molecule, has also been implicated in contact-dependent suppression in human MSCs [14, 17].

Our data from in vivo studies suggest that one mechanism by which feline ASCs decrease T cell-mediated inflammation is via the induction of CD8+ regulatory T cells [4]. Other groups have shown that CD137-CD137L co-stimulation can induce CD8+ regulatory T cells in the presence of IFN-γ [8, 18, 19]. Co-stimulation through the CD137-137L pathway also enhances suppressive T cell function and induces activated T cell anergy in human immune-mediated diseases [20, 21].

The purpose of this study was to define the mechanisms used by feline ASCs to suppress T lymphocyte proliferation, focusing on both soluble mediators and direct cell-cell contact ligands. Similar to human ASCs, we found that (1) feline ASCs induce G0–G1 cell cycle arrest in mitogen-activated T lymphocytes, (2) $PGE_2$ is a primary soluble factor partially responsible for the inhibition of T lymphocyte proliferation, and (3) a crucial ligand pair mediating feline ASC and T lymphocyte adhesion and secretion profile is ICAM-1/LFA-1. Notably, the increase in IFN-γ secretion induced after feline ASC-T cell direct interaction is abrogated when ICAM-1 is blocked. These findings shed light on both shared and unique aspects of feline ASC biology that may underscore how the administration of ASCs results in long-term reprograming of the immune system in cats with FCGS.

## Materials and methods

### Feline adipose-derived mesenchymal stem cells (ASCs)

Low passage (P1–P5) adipose-derived feline mesenchymal stem cells (ASCs) were isolated from subcutaneous feline adipose tissue surgically obtained from specific pathogen-free (SPF) cats or from client-owned cats undergoing routine surgery. Fat collection was conducted according to a protocol approved by the Institutional Animal Care and Use Committee, and the Clinical Trials Review Board, UCD (protocol number 18422). All owners of client-owned cats signed an informed consent form. All cats were free of feline immune deficiency virus and feline leukemia virus infection. ASC isolation and expansion was performed at the UC Davis William R. Pritchard Veterinary Medical Teaching Hospital Regenerative Medicine Laboratory, exactly as previously described [4].

### ASC culture and expansion

Feline ASCs were expanded as previously described [22]. In brief, cryopreserved ASCs were thawed in a 37 °C water bath and seeded into tissue culture flasks with Dulbecco's modified Eagle's medium (DMEM; Corning Life Sciences), 10% fetal bovine serum (HyClone Inc.), and 1% penicillin/streptomycin (Thermo Fisher Scientific) and incubated at 37 °C in 5% $CO_2$ at 90% humidity. Feline ASCs from passages 2–5 were used in the experiments. All ASC lines passed quality control assays including bacterial culture (all were sterile), high viability (> 90%), positive for CD90 (identity marker), negative for CD18 (purity marker), and negative for endotoxin and Mycoplasma.

### Peripheral blood monocular cell (PBMC) inhibition assay—mixed leukocyte reaction (MLR)

Feline ASCs were tested for their capability to inhibit lymphocyte proliferation with a mixed leukocyte reaction (MLR), carried out as previously described [4]. In brief, PBMCs were isolated from whole blood using gradient centrifugation and were co-incubated with irradiated ASCs in culture wells at a 1:5 (PBMC to ASC) ratio and activated with 5 mg/mL concanavalin A (ConA; Sigma-Aldrich). Cells were co-cultured for 4 days. Control wells included PBMCs alone and ConA-stimulated PBMCs. To determine indoleamine 2,3-dioxygenase (IDO) activity, the experiment was run as described; however, the media was supplemented with l-tryptophan (Sigma-Aldrich) to a final concentration of 600 μM. To measure proliferation, wells were spiked with 5-bromo-29-deoxyuridine (BrdU) at day 3 and

then cells were collected and processed per manufacturer's instructions (BrdU Flow Kit; BD Biosciences) at day 4.

Some protocols included the addition of antibodies to block TGF-β (10 μg/mL, anti TGF-β1 mouse monoclonal IgG, clone 9106, R&D systems), or interferon gamma (IFN-γ, 15 μg/mL, goat anti-feline polyclonal antibody #AF674, R&D systems), or chemicals to block prostaglandin E2 ($PGE_2$, 10 μm/mL, indomethacin, Cayman Chemical), or IDO (500 μM/mL 1-methyl-L-tryptophan (Sigma-Aldrich), a competitive inhibitor of tryptophan). Inhibitor concentration was determined by titration studies in our laboratory or based on previous publications [23].

### Feline ASCs and PBMC phenotyping

For the analysis of surface expression on feline ASCs and PBMCs, cells were harvested and resuspended at a concentration of $1 \times 10^6$ cell/mL in flow buffer (DPBS, 1% normal equine serum, 10 mM EDTA, and 0.1% sodium azide). Cells were incubated with antibodies for 30 min at room temperature. Antibodies included mouse anti-feline CD4-PE (clone 3-4F4, Southern Biotech), mouse anti-feline CD5-FITC (clone f43, Southern Biotech), mouse anti-feline CD8α-PE (clone Fe1.10E9, Leukocyte Antigen Biology Laboratory, UC Davis), mouse anti-human I-CAM 1 (CD54, clone MEM-111, Thermo Fisher Scientific), rat anti-mouse CD137L (clone TKS-1, Bio X Cell), polyclonal goat anti-human-PD-1 (cat#AF1086, R&D systems), polyclonal goat anti-human-PDL-1/B7-H1 (cat#AF156, R&D systems). The secondary antibody used for indirect labeling was R-Phycoerythrin $F(ab')_2$ Fragment donkey anti-goat IgG (Jackson ImmunoResearch Inc.) and Fluorescein Rabbit Anti-Rat IgG (Vector Laboratories). Cells were analyzed with a Beckman-Coulter Cytomics FC500 flow cytometer. Data analyses were done on Flowjo flow cytometry software (Tree Star, Inc.).

### Cell cycle analysis

Lymphocyte DNA content was determined with 7-amino-actinomycin D (7-AAD; BD Biosciences) incorporation to distinguish between lymphocyte populations in the S-phase, G1 phase, and G2–M phase in conjunction with BrdU incorporation (FITC BrdU Flow Kit; BD Biosciences). PBMCs were collected for 4 consecutive days from co-incubation experiments with feline ASCs. Cells were analyzed with a Beckman-Coulter Cytomics FC500 flow cytometer. Data analyses were done on Flowjo flow cytometry software (Tree Star, Inc.).

### Detection of intracellular IFN-γ

PBMCs were collected from the MLR on day 4 and resuspended in RPMI 1640 media with 10% heat-inactivated FBS, 1% Gluta-Max, 1 mM sodium pyruvate, 1% penicillin-streptomycin, 2 mM HEPES, 0.1% MEM NEAA, and 55 μM β-mercaptoethanol. Collected cells were stimulated with 25 ng/mL Phorbol-12-Myristate-13-Acetate (PMA, Sigma-Aldrich) and 500 ng/mL ionomycin (Sigma-Aldrich), treated with 1 μg/mL Brefeldin A and incubated for 3 h at 37 °C. Cells were then washed, stained with a viability dye (Fixable Viability Dye eFlour®780, eBioscience), fixed with 2% paraformaldehyde, permeabilized in wash buffer (DPBS with 0.5% bovine serum albumin, 0.1% saponin, and 0.02% sodium azide), and stained with anti-bovine IFN-γ-AlexaFluor647 antibody (clone CC302, Bio-Rad). Cells were analyzed with Beckman-Coulter Cytomics FC500 flow cytometer. Data analyses were done on Flowjo flow cytometry software (Tree Star, Inc.).

### Detection of secreted mediators

IDO, nitric oxide (NO), $PGE_2$, and IFN-γ were measured in the MLR culture supernatant collected on day 4. Supernatants were stored at − 80 °C until analyzed. $PGE_2$ and IFN-γ concentration were measured using commercially available feline-specific ELISA kits (Enzo Life Sciences and R&D systems, respectively), according to the manufacturer's instructions [8]. IDO activity was determined through the measurement of colorimetric kynurenine level assay, and NO was measured with a Griess reagent system (Griess Reagent System, Promega Corporation), both performed exactly as previously described [12]. All samples were read on a Synergy HT Multi-Mode microplate reader with Gen5 software (Biotek).

### MSC-PBMC static adhesion assay

Static adhesion assay was modified from Ren et al. [15]. In brief, feline ASCs were plated in 24-well plates ($5 \times 10^4$ cells/well) in 750 μL standard culture medium. Isolated PBMCs were fluorescently labeled with CellTracker™ Green CMFDA (5-chloromethylfluorescein diacetate, Thermo Fisher Scientific) and activated with 5 μg ConA for 1 h prior to adding to the ASCs ($1 \times 10^6$ cells/well). Cells were permitted to adhere to ASCs for 2 h at 37 °C in 5% $CO_2$. The plates were then rotated at 300 rpm for 5 min and washed with DPBS twice to remove non-attached PBMCs. Fluorescence was detected using a Synergy HT Multi-Mode microplate reader at 485-nm wavelength prior to and after washing to quantify the change in fluorescent intensity. The plate was also visualized and photographed on an inverted fluorescent microscope (EVOS FL, Thermo Fisher Scientific). In some experiments, blocking antibodies to ICAM 1 (anti-human CD54, clone MEM-111, Thermo Fisher Scientific), LFA-1 (anti-human clone R7.1, eBioscience), CD137 (anti-mouse clone 17B5, Bio X Cell), CD137L (anti-mouse clone TKS-1, Bio X Cell), PD-1 (anti-human PD-1, polyclonal goat IgG, R&D systems), or PDL-1 (anti-human PDL-1, polyclonal goat IgG, R&D systems) were added to determine which ligands mediated PBMC-ASC adhesion. The concentration

of antibodies used was determined by titration studies in our laboratory.

### Statistical analyses

Data analysis was performed using GraphPad Prism version 7 software (GraphPad Software). All experiments were performed with $n = 5$ (feline ASC lines and PBMCs donors) unless otherwise indicated. Statistical significance between two groups was determined by non-parametric Mann-Whitney-Wilcoxon test due to small sample size. $p$ values < 0.05 were considered statistically significant.

## Results

### Activated feline CD4+ and CD8+ T lymphocytes both secrete IFN-γ

Feline ASCs decrease activated T cell proliferation and secretion of pro-inflammatory cytokines, notably tumor necrosis factor alpha (TNF-α). However, unlike other species, including people, dogs, and horses, feline ASCs inhibit lymphocyte proliferation in the presence of increased IFN-γ concentration when ASCs are in direct contact with lymphocytes [6, 8, 12, 13, 24]. We previously hypothesized that feline ASCs could be licensed by IFN-γ and this signaling may be critical for the long-term reprograming of CD8+ regulatory T lymphocytes [25–27]. Our previous work did not identify the cell types responsible for IFN-γ secretion in our assays. As ASCs inhibit lymphocyte proliferation regardless of cell-cell contact, high IFN-γ concentration can be used as a surrogate marker of contact-mediated T cell inhibition and the reduction of IFN-γ secretion can be used as a marker of effective blockade of this pathway.

We found that feline CD4 and CD8 T lymphocytes both secrete IFN-γ after mitogen activation (Fig. 1a–d) and the secretion of IFN-γ from CD4+ T lymphocytes is significantly increased upon co-incubation with feline ASCs ($p = 0.02$; Fig. 1g), and the level of IFN-γ is sustained with a tendency to increase in CD8+ T lymphocytes in the presence of feline ASCs (Fig. 1h).

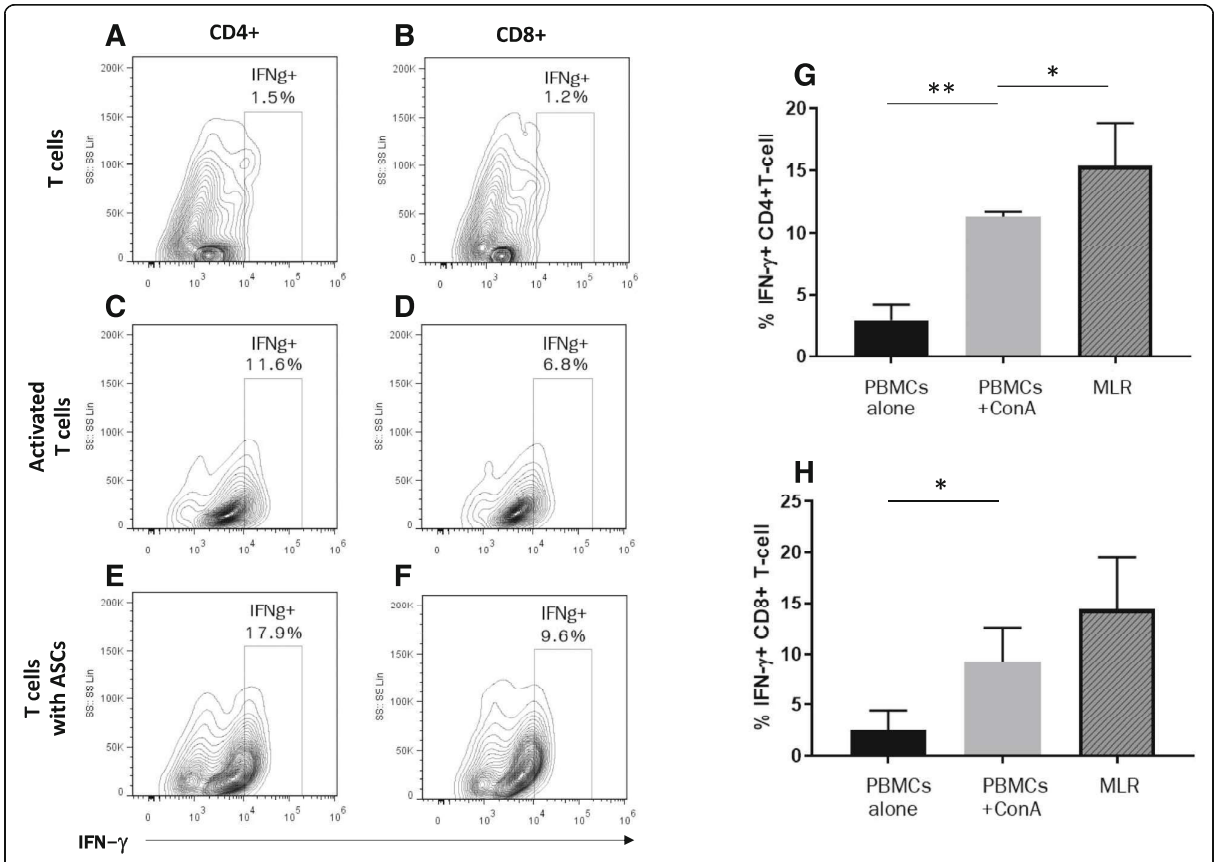


**Fig. 1** Both activated feline CD4 and CD8+ T cells secrete IFN-γ. Intracellular IFN-γ + cell population in **a** unstimulated CD4+ cells, **b** unstimulated CD8+ cells, **c** CD4+ cells stimulated with ConA, **d** CD8+ cells stimulated with ConA, **e** CD4+ cells in co-incubation with feline ASCs, and **f** CD8+ cells in co-incubation with feline ASCs. **g** Percentage of IFN-γ + CD4+ T cell increased after mitogen activation ($p = 0.008$) and was further augmented with feline ASC co-incubation ($p = 0.02$) **h** Percentage of IFN-γ + CD8+ T cell increased after mitogen activation ($p = 0.02$) with a trend to increase with feline ASC co-incubation, but was not statistically significant. Representative flow cytometric images and data from 5 independent experiments. *$p < 0.05$

### Feline ASCs decrease activated PBMC viability and inhibit lymphocyte proliferation through the induction of G0–G1 cell cycle arrest

Feline ASCs inhibit mitogen-activated T cell proliferation with and without the presence of cell-to-cell contact [8], but the mechanism of action is not known. Here we demonstrate that feline PBMC viability decreased upon mitogen activation ($p = 0.04$) and was even further exacerbated by the co-incubation with feline ASCs ($p = 0.008$; Fig. 2a–d). Additionally, cell cycle analysis revealed that the percentage of T lymphocytes in the G0–G1 phase increased with a concurrent decrease in the S-phase upon co-incubation with feline ASCs ($p = 0.03$). However, feline ASCs did not undergo increased apoptosis compared to the mitogen-activated condition (Fig. 2d–f). These findings suggest that feline ASCs inhibit activated PBMC viability and inhibit the proliferation of mitogen-activated T lymphocytes through the induction of G0–G1 cell cycle arrest.

### Feline ASC secretion of $PGE_2$ is partially responsible for inhibiting lymphocyte proliferation

Our previous data suggested that there are at least 2 mechanisms by which feline ASCs inhibit activated T cell proliferation, one relying on direct contact between the ASCs and T lymphocytes and another dependent on soluble factors [3, 8]. Feline ASCs constitutively produce low concentrations of immunomodulatory mediators in the absence of activation, but secretion is much higher in the presence of mitogen-activated T cells, particularly the secretion of IDO and $PGE_2$ which is enhanced by direct cell contact [8]. Although the feline ASC secretion profile has largely been determined [8], we wanted to (1) more fully elucidate mediators secreted by feline ASCs (in the presence and absence of contact) and (2) identify the soluble mediators critical for the inhibition lymphocyte proliferation, focused on TGF-β, IFN-γ, $PGE_2$, and IDO.

Nitric oxide (NO) may play an important role in human MSC-induced T lymphocyte immunosuppression [28]; however, feline ASCs did not secrete substantial quantities of NO even in the presence of activated T lymphocytes (Fig. 3a). Like $PGE_2$, IDO was secreted by activated feline ASCs and PBMCs [8] but only in the presence of direct cell-contact ($p = 0.008$; Fig. 3b).

We found that $PGE_2$ was partially responsible for the inhibition of activated lymphocytes by feline ASCs ($p = 0.03$); however, blocking TGF-β, IFN-γ, and IDO did not significantly restore lymphocyte proliferation (Fig. 3c–e). Blocking both $PGE_2$ and IDO demonstrated a slight increase, but did not significantly restore lymphocyte proliferation (Fig. 3e).

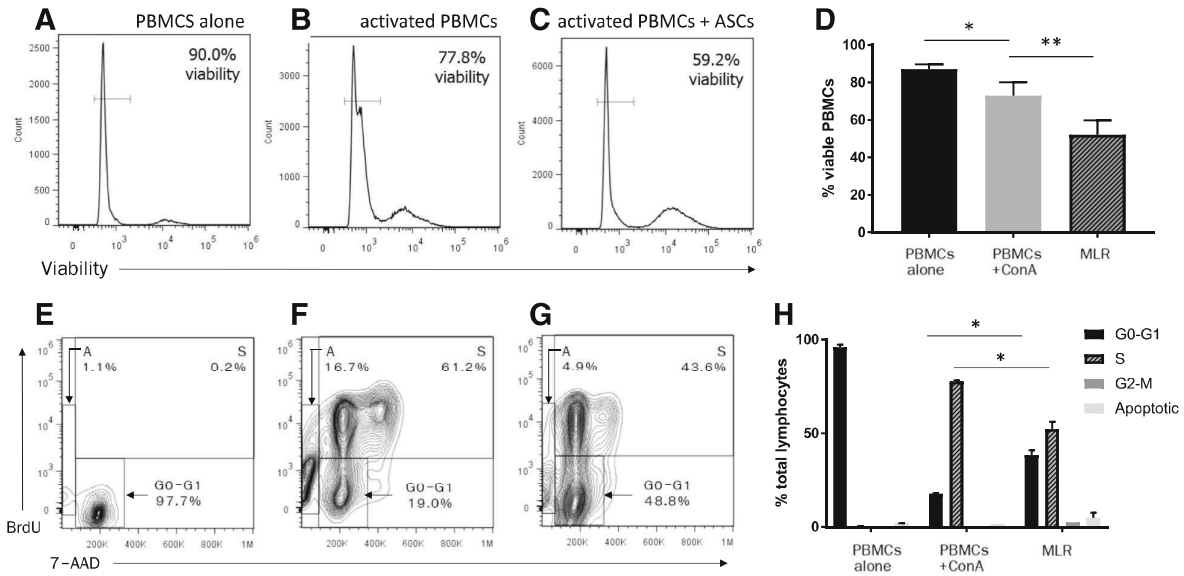


**Fig. 2** Feline ASCs decrease activated PBMC viability and induce cell cycle arrest in activated T lymphocytes**.** Representative images of flow cytometric analysis on day 4 from 5 MLR experiments with condition of **a** PBMCs only, **b** mitogen-activated PBMCs, and **c** PBMCs in co-incubation with feline ASCs. **d** the percentage of viable PBMCs decreased after mitogen activation ($p = 0.04$) and was further exacerbated by the co-incubation with feline ASCs ($p = 0.008$). Flow cytometric scatter plot of cell cycle analysis on T lymphocyte DNA content (7-AAD) and proliferation determined through BrdU incorporation of **e** PBMCs only, **f** mitogen-activated PBMCs, and **g** PBMCs in co-incubation with feline ASCs. **h** Percentage of T cells in the apoptotic, G0–G1, S, and G2–M phases from cell cycle analysis revealed that the percentage of T lymphocytes in the G0–G1 phase increased with a concurrent decrease in the S-phase upon co-incubation with feline ASCs ($p = 0.03$). *$p < 0.05$ **$p < 0.01$

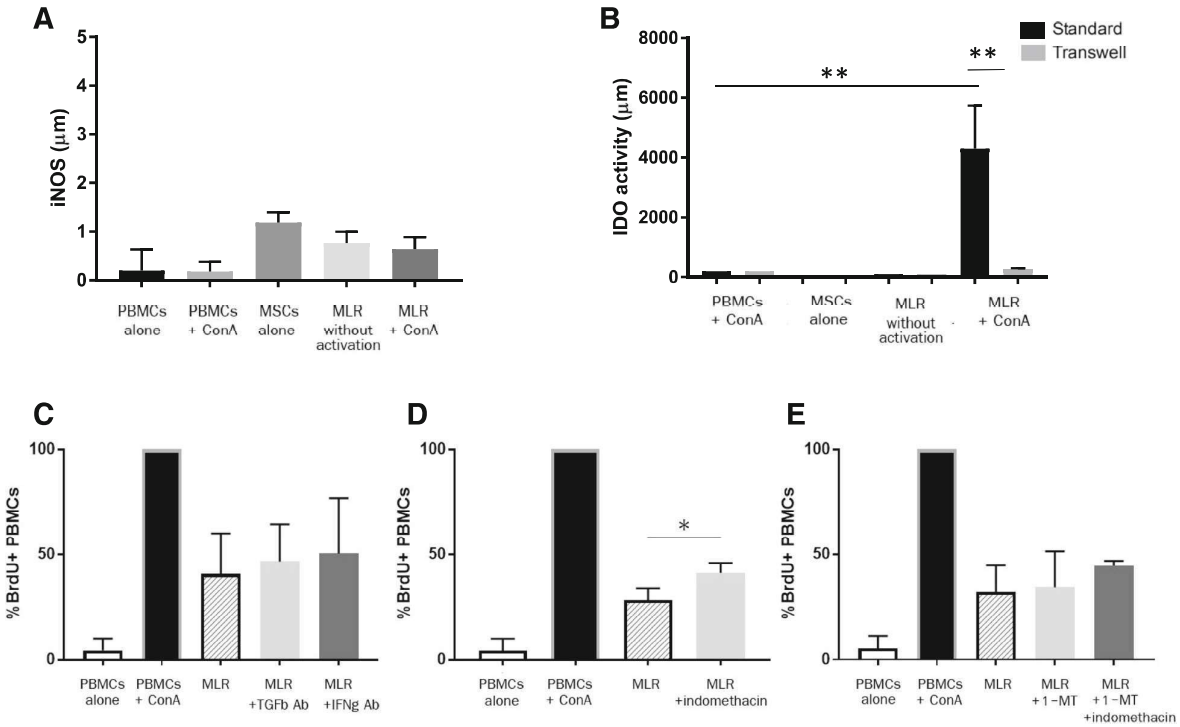


**Fig. 3** PGE2 secretion partially mediates feline ASC inhibition of T lymphocyte proliferation. **a** Measurement of iNOS production from supernatant of MLR on day 4 of co-incubation. Data from 5 independent experiments. **b** Measurement of IDO production from supernatant of MLR on day 4 of feline ASC and PBMC co-incubation with and without transwell. Data from 5 independent experiments. Degree of proliferation in the MLR with inhibition of soluble mediators: **c** addition of TGF-β and IFN-γ blocking antibody, **d** blocking of PGE2 with indomethacin, **e** blocking IDO with 1-methyltryptophan and PGE2 with indomethacin. Percentage of proliferation is normalized to 100% on mitogen activated condition for comparability. Blocking PGE2 significantly hindered the degree of suppression ($p = 0.03$). *$p < 0.05$, **$p < 0.01$

### ICAM-1 mediates the adhesion between feline ASCs and T lymphocytes

Cell-cell contact is an important factor for MSC-mediated T cell immunosuppression [12, 17, 29]. Given the importance of cell-cell contact and the unique contact-dependent mediator secretion profile for feline ASCs in particular, we investigated the potential role of 3 ASC cell surface receptors [CD54 ICAM-1, PDL-1, and CD137L] to regulate T cell-feline ASC adhesion. We first determined if ICAM-1, PDL-1, and CD137L were expressed on feline MSCs and whether co-incubation of activated T cells with MSCs resulted in increased expression of these surface receptors. Flow cytometric analysis revealed that activated feline ASCs expressed ICAM-1, CD137L, and PDL-1 on their surface (Fig. 4a–c). Activated feline T cells express LFA-1 [30], PD-1 [31], and CD137 [unpublished data]; however, it was unknown if, similar to human MSCs, ASC co-incubation with activated T cells would decrease PD-1, CD137, and LFA-1 expression on activated T cells. We found that, unlike human MSCs, feline ASCs did not decrease PD-1 expression on activated T cells (Additional file 1).

We then utilized blocking antibodies against ICAM-1/LFA-1, CD137/CD137L, and PD-1/PDL-1 to directly test whether these ligand pairs mediated feline ASC-lymphocyte adhesion in static conditions. Neither the blockade of CD137/CD137L nor PD-1/PDL-1 significantly altered T cell-ASC adhesion (Fig. 4d–j). However, blocking ICAM-1 significantly reduced T cell-ASC adhesion to levels comparable to adhesion between non-activated T cells and ASCs ($p = 0.045$; Fig. 5a–d). Blocking ICAM-1 also resulted in a concurrent significant reduction of IFN-γ secretion ($p = 0.002$; Fig. 5e). These findings collectively suggest that ICAM-1 is important for mediating the adhesion between feline ASCs and T cell and may be involved in contact-dependent immunomodulation by feline ASCs.

## Discussion

Cats are increasingly used as translational models for MSC-based therapies, and a number of inflammatory feline diseases resemble human inflammatory conditions [32, 33]. Feline ASCs have been used in a number of clinical trials for diseases including feline chronic gingivostomatitis (FCGS), chronic enteropathy, chronic kidney

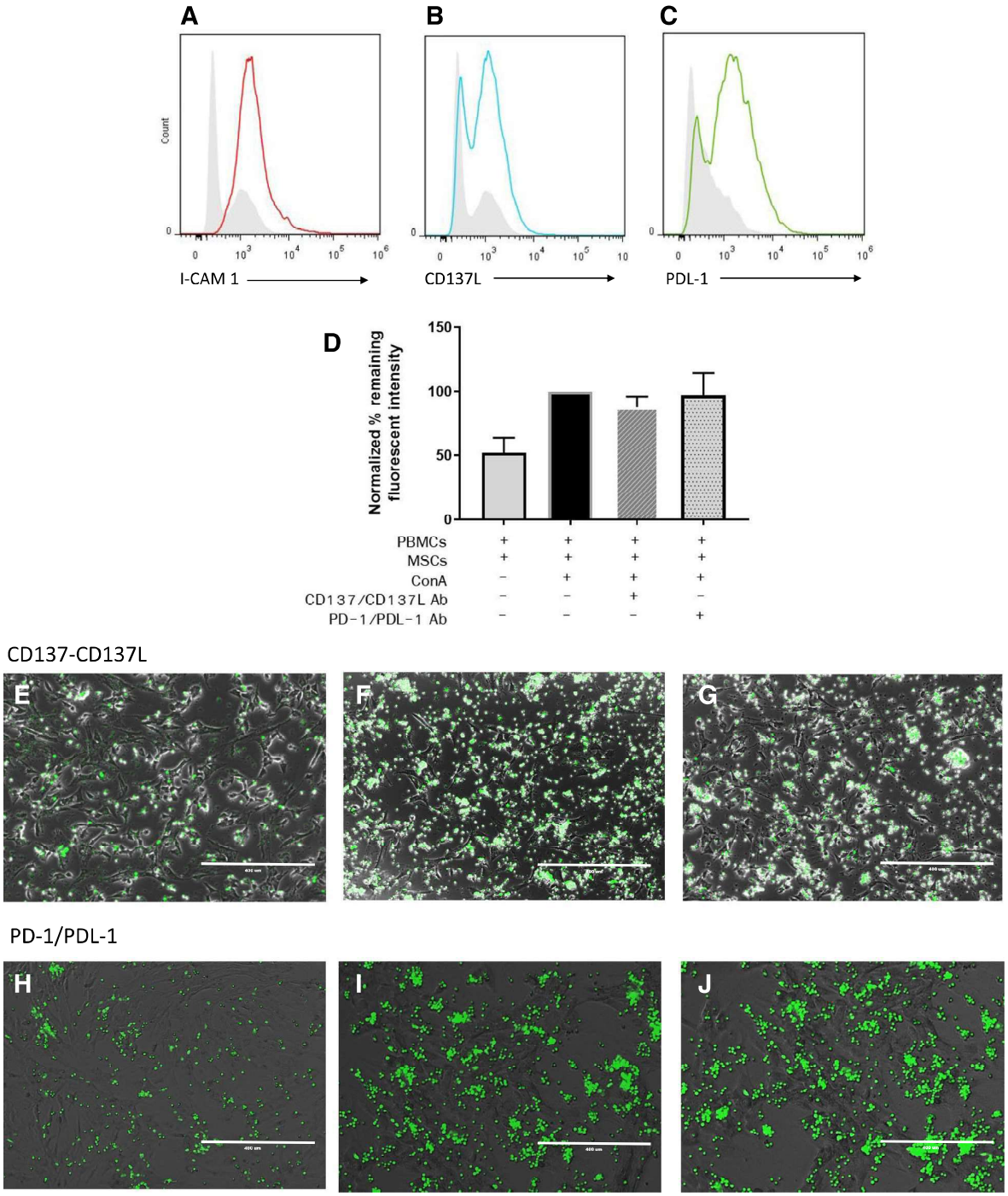


**Fig. 4** ICAM-1, CD137L, and PDL-1 are all expressed on activated feline ASCs; however, CD137L and PDL-1 do not mediate MSC-T cell adhesion. Expression of **a** ICAM-1, **b** CD137L, and **c** PDL-1 ligands on activated feline ASCs. Gray histogram indicated background fluorescence of unstained samples. **d** Percentage of remaining fluorescence intensity from CMFDA-labeled PBMCs after removal of non-adherent cells from static adhesion assay after the addition of CD137/CD137L and PD-1/PDL-1 blocking antibodies. Data is normalized to 100% on a standard MLR condition for comparability. Fluorescent images of static adhesion assay demonstrating adherent lymphocytes to ASCs from **e**, **h** non-activated MLR, **f**, **i** stimulated MLR with ConA, and **g**, **j** stimulated MLR with ConA and addition of CD137/CD137L and PD-1/PDL-1 blocking antibodies respectively. Scale bar = 400 μm. Representative flow analysis images in **a**–**c** from 3 independent experiments. Data gathered in **d** from 5 independent experiments

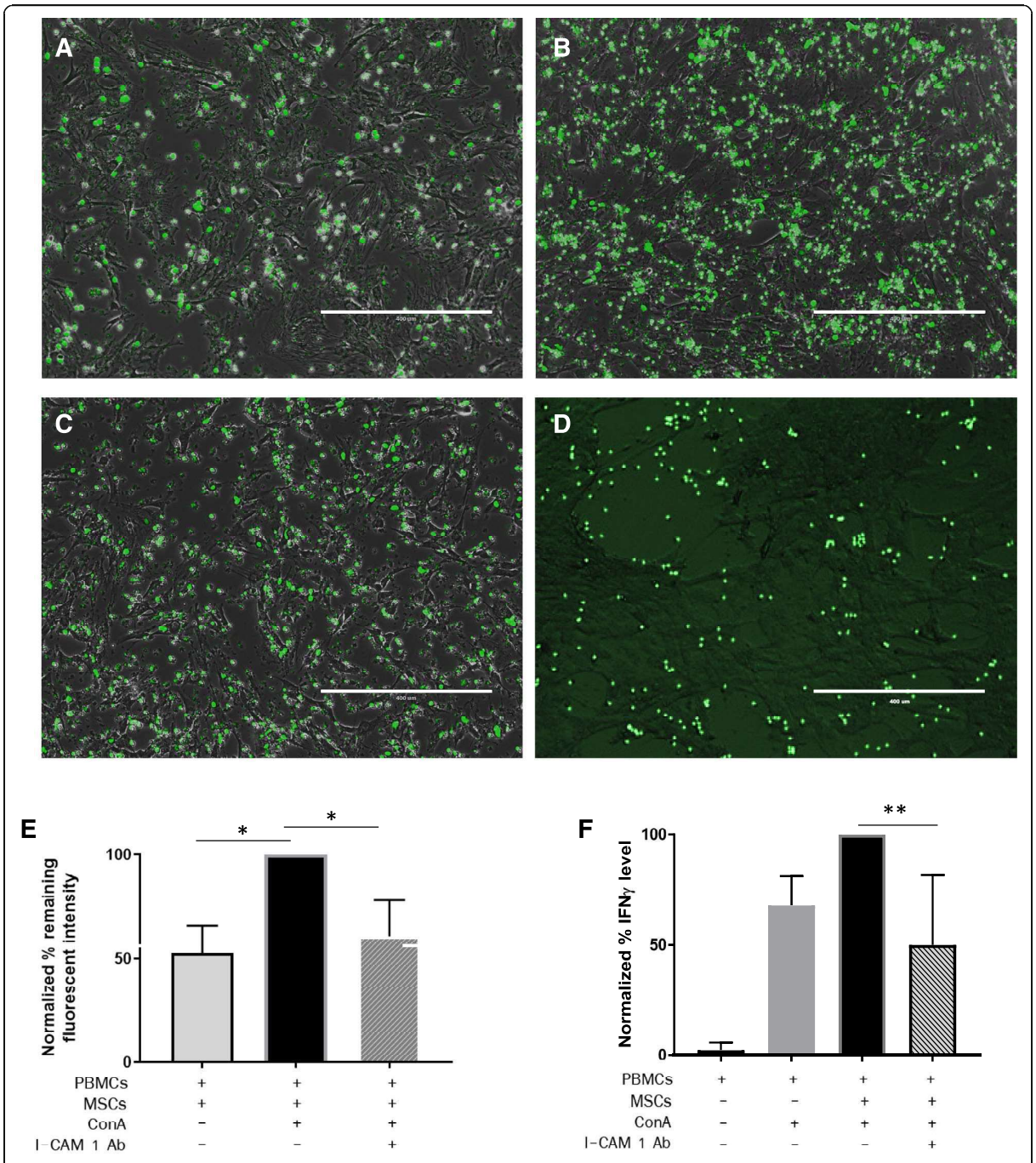


**Fig. 5** I-CAM 1 ligand mediates the adhesion between feline ASCs and T lymphocytes. Fluorescent images of static adhesion assay demonstrating adherent lymphocytes to feline ASCs from **a** non-activated MLR, **b** stimulated MLR with ConA, **c** stimulated MLR with ConA and addition of ICAM-1 blocking antibodies, and **d** stimulated MLR with ConA and addition of ICAM-1/LFA-1 blocking antibodies. Scale bar = 400 μm. **e** Percentage of remaining fluorescence intensity from CMFDA-labeled PBMCs after removal of non-adherent cells from static adhesion assay with added condition of ICAM-1 blocking antibodies. Data is normalized to 100% on standard MLR condition for comparability. **f** Changes in IFN-γ concentration in the MLR after addition of ICAM-1 blocking antibodies. Data from 5 experiments, normalized to 100% on standard MLR condition for comparability. *$p < 0.05$, **$p < 0.01$

disease, and feline asthma with varying degrees of success [3, 4, 34–36]. However, the exact mechanism(s) by which feline ASCs alter T cell responses remain vaguely understood. The objective of this study was to elucidate the underlying pathways utilized by feline ASCs to mitigate inflammatory conditions characterized by activated T cell proliferation.

MSCs can modulate T cell function, suppress T cell proliferation, and decrease T cell viability, but the mechanisms by which they accomplish these tasks are different between species and tissue sources. Based on our current study, we determined that feline ASCs inhibit T cell proliferation via cell cycle arrest in the G0–G1 phase, similar to murine BM-MSCs [37], equine BM- and cord blood-derived MSCs [38], and canine ASCs (unpublished data). MSCs from other tissues sources, including equine ASCs and cord tissue-derived MSCs inhibit T cell proliferation through induction of apoptosis [38]. Human MSCs cause T cell apoptosis through a pathway mediated by IDO and IL-10 [39, 40]. Despite the species and tissue source variation, mechanisms underlying MSC inhibition of T cell responses are mediated by soluble factors and/or direct cell-to-cell interactions.

The interaction of cell surface receptors and their respective ligands on target cells are crucial for cell communication and modulation of cell functions [41]. ICAM-1 is an inducible cell adhesion glycoprotein expressed on the surface of a wide variety of cell types, including MSCs across different species [42]. ICAM-1 interactions with the β2 integrin CD11a/CD18 (LFA-1) on the surface of lymphocytes are functionally important as costimulatory molecules for T cell activation [43]. In humans, ICAM-1 is constitutively expressed at a low level on the MSC surface but is significantly unregulated in the presence of pro-inflammatory cytokines, such as IFN-γ [44, 45]. Here we demonstrate that feline MSCs express ICAM-1 on their surface and that this molecule is similarly upregulated by activation. Further, our data demonstrate that this ligand plays a critical role in ASC-lymphocyte adhesion and signaling.

Although feline ASCs are capable of inhibiting lymphocyte proliferation in the absence of direct cell contact [8], the secretion profile of ASCs with and without direct cell-cell contact is very different. Our current data demonstrate that ICAM-1/LFA-1 interaction is critical for cell-cell adhesion and plays an important role in the secretion of immunomodulatory mediators, from both T cells (IFN-γ) and MSCs ($PGE_2$), as blocking these ligands significantly reduced their concentration. Our findings mimic in vivo findings in a mouse model where blockade of ICAM-1 ligand also decreased IFN-γ secretion and reduced pulmonary barrier damage in T cell-induced acute lung injury [46]. In mice, it was also found that the overexpression of ICAM-1 on MSCs can enhance the immunosuppressive effects of MSCs, including modulating T cell responses, dendritic cell maturation, and secretion of immunomodulatory soluble factors in vitro [47].

We also evaluated the ligand pairs CD137-CD137L and PD-1/PDL-1. CD137 (4-1BB), an inducible protein expressed on both CD4+ and CD8+ T cells, is functionally involved in signaling T cell proliferation [48]. CD137-CD137L interaction has been implicated as one potential immunosuppressive mechanism used by human MSCs in the treatment of multiple sclerosis [49]. CD137-CD137L interaction has also been implicated for the paradoxical increase in IFN-γ that supports CD8 T regulatory expansion [50]. Similarly, programmed death-1 (PD-1) and its ligand, PD-L1, is an important inhibitory pathway of T cell response and has been implicated as a crucial interaction used by human MSCs to inhibit T cell responses [14, 51]. However, our data suggest that although CD137L and PDL-1 are expressed on activated feline ASCs, they are not the primary mediators of ASC T cell adhesion and do not mediate IFN-γ secretion in vitro.

Upon activation, feline ASCs secrete several immunomodulatory mediators, including IDO, $PGE_2$, IL-6, IL-8, and TGF-β [8, 29, 52]. However, the principal immunomodulatory mediators used by MSCs appear to vary by species. Human MSCs primarily utilize IDO whereas canine MSCs, both bone marrow-derived and adipose-derived, utilize TGF-β and $PGE_2$ to suppress lymphocyte proliferation [53–55]. With feline ASCs, we found that blocking IDO, with 1-methyltryptophan, or adding a TGF-β blocking antibody to the assay did not significantly alter T cell proliferation. Like dogs and horses, $PGE_2$ is at least one soluble factor utilized by feline ASCs to block T cell proliferation as blocking $PGE_2$ with indomethacin, a competitive inhibitor of $PGE_2$, partially restored T cell proliferation [12, 53]. However, the role of $PGE_2$ was modest compared to similar experiments conducted with equine ASCs [38], implying that other soluble factors are also likely involved in feline ASC-T cell interaction. Despite a trend, blocking both $PGE_2$ and IDO did not significantly restore T cell proliferation likely due to small sample size and the variability of T cell responses to mitogens from different cat donors.

Our findings are in agreement with data from others that used a different $PGE_2$ inhibitor, NS-398, to reverse the immunosuppressive effects of feline ASCs [52]. Although nitric oxide (iNOS) is implicated in the mechanism of MSC-mediated T cell suppression by both human and murine MSCs [28, 56], we found that feline ASCs do not produce a substantial amount of iNOS, either with or without activation. These findings correspond to a recent study where the level of iNOS RNA in feline ASCs was low or undetectable [29].

$PGE_2$ is an eicosanoid lipid mediator which is produced by MSCs from most species, including human, murine, canine, equine, and feline [13, 57]. Although

$PGE_2$ may be pro-inflammatory in some contexts, it can also be immunosuppressive and is capable of decreasing IL-2 production from T cells and shifting CD4+ T cells from a predominantly cytotoxic Th1 response to a more balanced Th2/Th17-mediated response [58]. $PGE_2$ also promotes the development of regulatory T cells and mediates their suppressive actions on effector T cells [59, 60]. These mechanisms may partially explain how feline ASCs are successfully used to treat FCGS, an immune-mediated inflammatory disease.

MSCs generally require licensing with IFN-γ to exert their immunosuppressive effects [61], our study showed that IFN-γ is produced by both CD4+ and CD8+ T lymphocytes upon mitogen activation and the production of IFN-γ from both T cell subsets is further enhanced by feline ASCs. These findings indicate that feline ASCs may be appropriate for therapeutic trials for both CD4+- and CD8+-mediated alloreactive diseases.

## Conclusion

Feline ASCs utilize $PGE_2$ and ICAM-1/LFA-1 ligand interaction to inhibit T cell proliferation by causing cell cycle arrest in the G0–G1 phase. While many questions remained to be addressed, these findings provide a deeper understanding of the underlying mechanisms involved in the immunosuppression by feline ASCs and will lead to more efficient implementations of ASC-based therapy for the modulation of immune-mediated inflammatory disease models.

## Additional file

**Additional file 1: Figure S1.** Feline ASCs do not downregulate PD-1 expression on activated PBMCs. Flow analysis on PD-1 expression on activated T lymphocytes with and without co-incubation with feline ASCs. Representative image of flow cytometry analysis from 3 different MSC lines. (DOCX 185 kb)

### Abbreviations

ASC: Adipose-derived mesenchymal stem cell; BrdU: 5-Bromo-29-deoxyuridine; ConA: Concanavalin A; DPBS: Dulbecco's phosphate buffered saline; ELISA: Enzyme-linked immunosorbent assay; FBS: Fetal bovine serum; IDO: Indoleamine 2,3 dioxygenase; IFN-γ: Interferon gamma; MLR: Mixed leukocyte reaction; MSC: Mesenchymal stem cell; PBMC: Peripheral blood monocular cell; $PGE_2$: Prostaglandin E2; SPF: Specific pathogen free; TGF-β: Transforming growth factor beta; TNF-α: Tumor necrosis factor alpha

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### Authors' contributions

NT is responsible for the collection and assembly of data, data analysis and interpretation, and manuscript writing. SI is responsible for the collection and assembly of the data and review of the manuscript. NJW is responsible for the collection and assembly of the data and review of the manuscript. BA is responsible for the provision of the study material and review of the manuscript. DLB is responsible for the conception and design, data analysis, financial support, manuscript writing, and final approval of the manuscript. All authors read and approved the final manuscript.

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### Availability of data and materials

All datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Ethics approval and consent to participate

Feline ASCs were obtained according to a protocol approved by the Institutional Animal Care and Use Committee, and the Clinical Trials Review Board, UCD (protocol number 18422). All owners of client-owned cats signed an informed consent form.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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## Publisher's Note

# Chapter 3

## Feline adipose-derived mesenchymal stem cells induce effector phenotype and enhance cytolytic function of CD8+ T-cells

**Authors:** **Nopmanee Taechangam, Naomi Walker, Dori Borjesson**

**Abstract**

Feline adipose-derived mesenchymal stem cells (ASCs) engage with a variety of immune cells and have been used in several clinical trials for the treatment of inflammatory and immune-dysregulated diseases in cats, but the impact they exert on the functional characteristics on T-cells, particularly CD8+ T-cells, remain to be elucidated. Modified mixed leukocyte reaction was performed between feline ASCs and PBMCs. Changes of cell cycle stages, phenotype and cellular senescence were determined through flow cytometry and gene expression analysis. Cytotoxicity assay was performed to evaluate CD8+ T-cell effector function. Feline ASCs induce cell-cycle arrest on CD8+ T-cells in a contact-dependent manner, downregulate CD8 surface expression, shift their phenotype toward terminally differentiated effector cells (CD57+, CD45R+, CD62L-). CD8 T-cells interacted with feline ASCs also upregulated granzyme B, IL-2 and KLRG-1 expression and have enhanced cytotoxic potential, evident by the increased percentage of lysis on target cells. Our findings suggest that feline ASCs 1) alter CD8+ T-cells towards terminally differentiated, proinflammatory effector phenotype with limited proliferative capacity, and 2) enhance their cytotoxic potential through granzyme B upregulation. These cytotoxic CD8+ T-cells could aid in disease cure in cases caused by an underlying, unresolved viral infection.

## Introduction

Feline adipose-derived mesenchymal stem cells (ASCs) engage with a variety of immune cells and have been used in several clinical trials for the treatment of inflammatory and immune-dysregulated diseases with varying degrees of success [1-4]. However, the mechanisms by which feline ASCs can sustainably alter the immune response remain to be elucidated.

Feline ASCs have effectively treated cats with feline chronic gingivostomatitis (FCGS) [1, 2]. FCGS is a painful, debilitating oral disease with severe inflammation of the gingival tissue. The disease is difficult to successfully manage with standard medical or surgical interventions [5]. Although the cause is multifactorial, FCGS is characterized largely by a persistent inflammatory response from inappropriate T-cell activation and tissue infiltration, predominantly with B cells and CD8+ T-cells [6]. Underlying viral infection (or antigenic stimulation), particularly secondary to feline calicivirus (FCV), has been implicated [7, 8]. The outcomes of feline ASC interaction with CD8+T-cells have not been explored.

T-cell fate decisions are regulated by environmental signals, including growth factors, cytokines, and cell–cell contact. ASCs can regulate T-cells through secretion of soluble factors and through direct ligand cell-cell contact. We previously identified a primary soluble mediator, $PGE_2$, and a critical ligand interaction, ICAM-1/LFA-1 as necessary for feline ASC inhibition of T-cell proliferation via the induction of G0-G1 cell cycle arrest *in vitro* [9]. Based on the withdrawal of T-cells from cell cycle progression, feline ASCs may induce cellular senescence or terminal differentiation.

Our study demonstrated that feline ASCs induce cell cycle arrest in CD8+ T-cells in a contact dependent mechanism, alter CD8+ T cell phenotype to terminally differentiated effector cells and augment their cytotoxic function through upregulation of granzyme B.

## Materials and Methods

### Adipose-derived Mesenchymal Stem Cell Collection and Culture

Adipose-derived feline mesenchymal stem cells (ASCs) were isolated from subcutaneous fat surgically obtained from specific pathogen-free (SPF) cats or from client-owned cats undergoing routine surgery. All owners of client-owned cats signed an informed consent form. All cats were free of feline immune deficiency virus (FIV) and feline leukemia virus (FeLV) infection. ASC isolation and expansion was performed as previously described [10]. Briefly, cryopreserved ASCs were thawed in a 37°C waterbath, seeded into tissue culture flasks with Dulbecco's modified Eagle's medium (DMEM low glucose; GIBCO, Grand Island, NY), 10% fetal bovine serum (FBS, Atlanta Biologicals, Flowery Branch, GA), and 1% penicillin/streptomycin (GIBCO) and incubated at 37°C in 5% $CO_2$ at 90% humidity.

Feline ASC lines were characterized by their surface protein expression using flow cytometry. All ASC lines passed quality control assays including bacterial culture (all were sterile), high viability (>90%), positive for CD90 (identity marker), negative for CD18 (purity marker) and negative for endotoxin and Mycoplasma. All antibodies were purchased from the Leukocyte Antigen Biology Laboratory, University of California, Davis (UCD), unless otherwise indicated. Antibodies included MHC II (42.3), CD18 (FE3.9F2), CD90 (CA1.4G8), CD44 (IM7; BioLegend), and CD105 (SN6; eBioscience). For unconjugated antibodies, a mouse IgG-

phycoerythrin antibody (Jackson ImmunoResearch Laboratories) was used for secondary labeling. Canine CD8a (CA9.JD3), rat immunoglobulin G-allophycocyanin (IgG-APC) (eBR2a; eBioscience), and mouse IgG-APC (MCA928; AbD Serotec) were used as isotype controls. Feline ASC lines were obtained from a total of 6 animals between the age of 4.5-11 year-old from 3 spayed females and 3 castrated males. Low passage (P2-P5) ASCs were used for experiments.

**Modified Mixed Leukocyte Reaction (MLR)**

Feline ASCs were co-incubated with freshly enriched peripheral blood mononuclear cells (PBMCs) with and without the addition of the mitogen ConcavalinA (ConA, 5 µg/mL Sigma-Aldrich, St. Louis, MO) at 1:10 ratio between ASCs and PBMC, as previously described [2]. PBMCs were obtained from 12 healthy colony cats between the age of 2-8 years old used for nutritional study at UC Davis. Four conditions of co-cultures were performed for each experiments in duplicates; PBMCs alone, PBMCs with ConA, PBMCs with ASCs without ConA and PBMCs with ASCs with ConA. Changes were assessed at 96 hours, except for gene expression studies with additional 24 hours. Experimental design after performing an MLR is outlined in Figure 1.

**T-Lymphocyte Phenotyping**

PBMCs were harvested at 96 hours after MLR and resuspended at a concentration of 1 x $10^6$ cell/mL in flow buffer (DPBS, 1% normal equine serum, 5 mM EDTA and 0.1% sodium azide). Cells were incubated with antibodies for 30 minutes at room temperature. Antibodies included: mouse anti-feline CD8-PE (clone Fe1.10E9, Leukocyte Antigen Biology Laboratory, UC Davis) – 1

μl, anti-feline CD25-FIT C (clone 9F23, a gift from K. Ohno, Tokyo, Japan) – 0.2 μl, human anti-mouse CD45R/B220 – APC-Cy7 (clone RA3-6B, BioLegend, San Diego, CA) – 1.25 μl, human anti-mouse CD57- FIT C (clone TB01, ThermoFisher, Waltham, MA) – 1 μl, and human anti-mouse CD62L-APC (clone LAM1-116, MyBiosource.com) – 2.5 μl. Reactivity of anti-mouse antibodies against feline T-cells were determined by previously reported studies. Multicolor flow cytometry was performed and cells were analyzed with a Beckman-Coulter Cytomics FC500 flow cytometer with CXP software (Hialeah, FL. Data were analyzed on FlowJo flow cytometry software (Becton Dickinson, San Jose, CA). Cells were pre-gated on CD8+ before further analysis and single-color beads were used for compensation.

**Cell Cycle Analysis**

Cell cycle analysis was performed as previously described [9]. In brief, PBMCs were incubated with both 7-amino-actinomycin D (7-AAD; BD Biosciences, San Jose, CA), to determine DNA content, and with BrdU, to determine proliferation (FITC BrdU Flow Kit; BD Biosciences) and to distinguish between S-phase, G1 phase and G2-M phase. PBMCs were collected at day 4 from co-incubation experiments with feline ASCs with and without transwell insert. Cells were analyzed with a Beckman-Coulter Cytomics FC500. Data analyses were done on FlowJo flow cytometry software.

**CD8+ T-cell Enrichment**

PBMCs were harvested from the MLR at various time-points according to experimental set-up and resuspended in DPBS (Figure 1). Cells were filtered through a 35 μm cell strainer cap

(BD Biosciences), washed (400x g, 5 minutes), and resuspended in MACs buffer (DPBS (GIBCO), 0.5% bovine serum albumin (Fisher Scientific, Santa Clara, CA), 2 mM EDTA (Sigma)). Cells were labeled for CD8 followed by anti-mouse IgG Microbeads (Miltenyi Biotec (San Diego, CA)) and run through a MACS MS separation column (Miltenyi Biotec) per manufacturer's recommendation to obtain a positively selected CD8+ T-cell population.

**Senescence Associated-β Galactosidase (SA-β-Gal) Activity**

Detection of SA-β-Gal activity was performed with CellEvent™ Senescence Green Flow Cytometry Assay Kit (ThermoFisher Scientific) according to manufacturer's instruction. Briefly, PBMCs incubated in a standard MLR for 4 days, then transferred to a secondary culture for another 6 days before were harvesting on day 10, surface stained with anti-CD8 antibodies in flow buffer for 30 minutes, fixed in 2% paraformaldehyde for 10 minutes and incubated with X-Gal substrate for 2 hours at 37°C in the absence of $CO_2$. The labeled cells were then analyzed on a flow cytometer (Cytomics FC500) using a 488-nm laser.

**Cytolytic assay – Lactate Dehydrogenase (LDH) Enzyme Release Assay**

Crandell-Reese feline kidney cells (CRFK; target cells) were plated in a 96-well tissue culture plate overnight (phenol-red free Dulbecco's modified Eagle media (DMEM) and 5% FBS to minimize background interference for colorimetric assay) and infected with Feline Calcivirus (FCV, Kaos strain, generously provided by Dr. Patty Pesavento) at MOI = 0.01. The MOI and infection kinetics of FCV was pre-determined by titrating for an infectious dose that did not create cytopathic effects within the timeframe of the assay. FCV was allowed to infect CRFK

cells for 1.5 hours and then isolated CD8+ T-cells were co-incubated with the infected target cells for 4 hours at 2:1 ratio. CD8+ T-cells were co-incubated for four days with ASCs ("primed") and without ASCs in the presence and absence of activation prior to target cell interaction. After 4 hours, the supernatant was collected and LDH activity was determined with CyQUANT™ LDH Cytotoxicity Assay Kit (Invitrogen, Carlsbad, CA) per manufacturer's instructions and read on a microplate reader at 490 nm and 680 nm (Spectra Max 340; Molecular Devices, San Jose, CA). The cytotoxicity assay was modified according to Weidmann et. al [11].

**Table 1:** List of primers used for gene expression

| Target Gene | Accession Number | Sequence | Product size (bp) |
|---|---|---|---|
| **GAPDH** | NM_001009307.1 | Fw: 5'-CCATGTTTGTGATGGGCGTG-3'<br>Rev: 5'-TGATGGCATGGACTGTGGTC-3' | 159 bp |
| **IL-2** | NM_001043337.1 | Fw: 5'-GCAATTACTGCTGGATTTACGGTTGC-3'<br>Rev: 5'-AGTCAGCGTTGAGAAGATGCTTTG-3' | 358 bp |
| **Granzyme B** | XM_006932823.3 | Fw: 5'-CCACCCAGACTATAATCCAAAGAA-3'<br>Rev: 5'-CAGTCAGCTTGGCCTTTTTCA-3' | 77 bp |
| **Perforin** | NM_001101660.1 | Fw: 5'-GGGAGCGCTTTTCCGAAATAG-3'<br>Rev: 5'-CTGGAAGTTCAT GACCTCCA-3' | 380 bp |
| **KLRG-1** | XM_023256758 | Fw: 5'-AGGAAATGAGCCTGCTTCAA-3'<br>Rev: 5'-CCGTAAAAGAGCCTCACAGC-3' | 194 bp |

Fw: Forward primer, Rev: Reverse primer

## Gene Expression

PBMCs or enriched CD8+ T-cells were preserved in RLT buffer and RNA was extracted (RNeasy mini kit, Qiagen, Germantown, MD) and stored at -80°C until cDNA was synthesized (High-Capacity cDNA Reverse Transcription Kits, Applied Biosystems, Foster City, CA) per manufacturers' instructions and stored at -20°C until analysis. Quantitative PCR (qPCR) was performed using Power SBYR green (Applied Biosystems) on a QuantStudio 3 Real-Time PCR system (Thermo Fisher). KLRG-1 primers were designed using Primer 3 Plus [12] with sequences from Genebank. Feline perforin, Granzyme B and IL-2 primer sequences were previously

published [13, 14]. Primer information is provided in Table 1. GAPDH was used as housekeeping gene. Activated CD8+ T-cells alone was used as reference sample. All samples were run in triplicates. Changes in gene expression were calculated by the ΔΔCT method and depicted as fold change in gene expression compared to control at 24 and 96 hours time points as depicted in Figure 1.

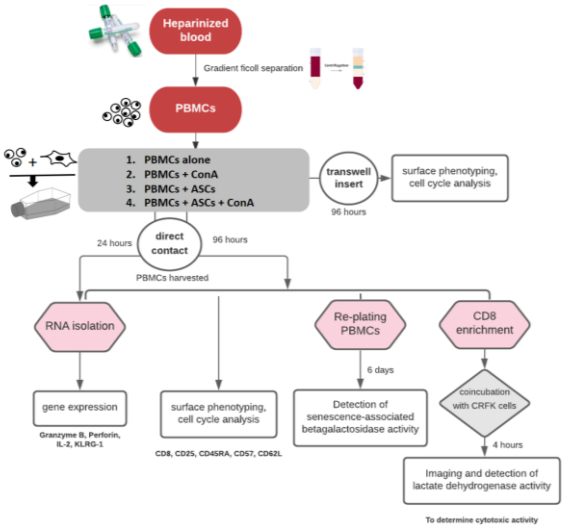


**Figure 1: Study Design** Image presents the experimental design and timepoints performed. PBMCs were typically harvested at 96 hours except for an additional 24 hours timepoint for gene expression study. Abbreviations: PBMCs – peripheral blood mononuclear cells, CRFK cell – Crandell-Reese feline kidney cells, ConA – Concanavalin A

**Statistical Analyses**

Normal distribution of the data was tested using the Shapiro-Wilk test. A paired t-test (normally distributed data) or Mann–Whitney U test (non-normally distributed data) was used to determine differences in between two groups in data set. Difference between 3 groups was determined using Kruskal-Wallis test with Dunn's multiple comparison post-test. A commercially available statistical software was used for all statistical analyses (GraphPad InStat version 3.06 for Windows; GraphPad Prism, San Diego, CA). A P value of <0.05 was considered statistically significant.

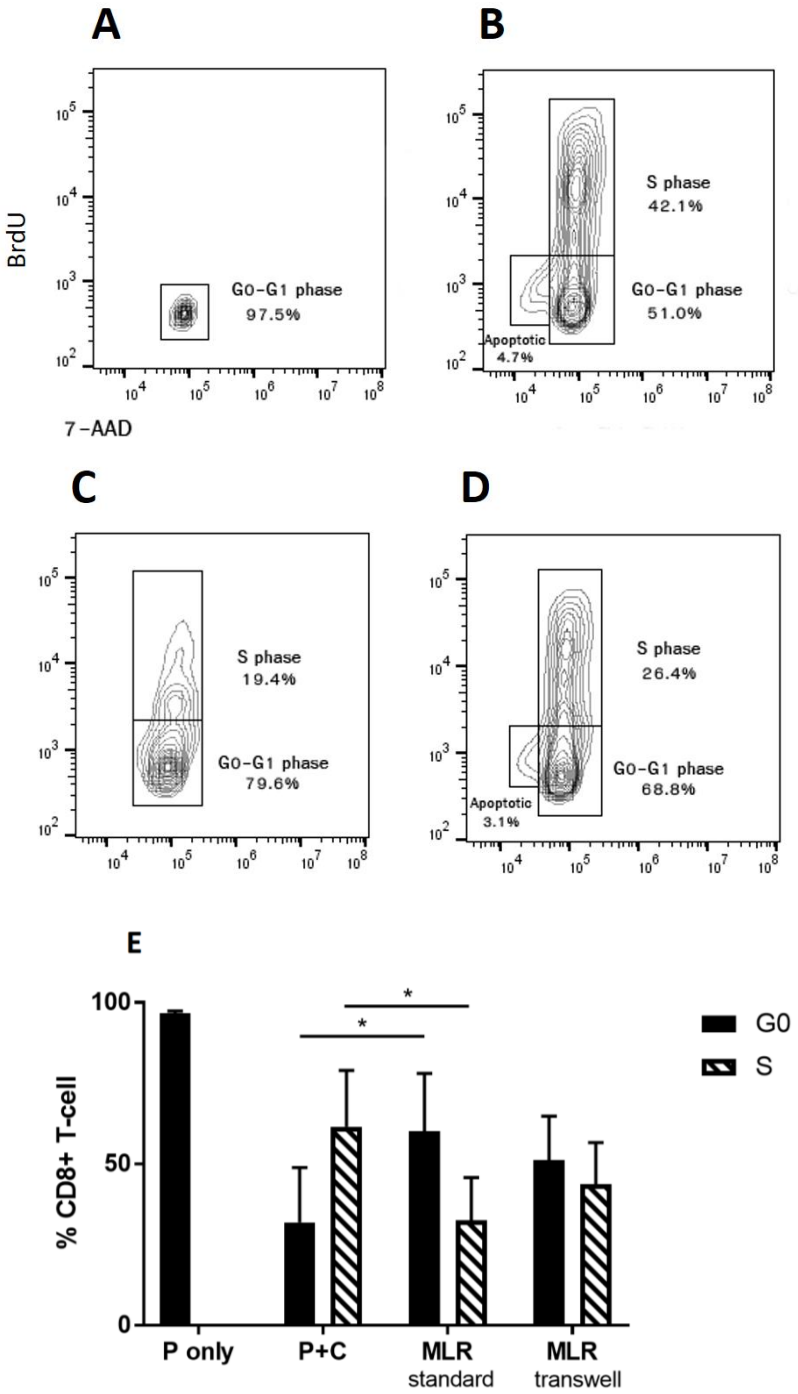


**Figure 2: Feline ASCs induce cell cycle arrest in CD8+ T-cells in a contact-dependent manner.** Representative flow-plot on cell cycle analysis with and without transwell (A) resting CD8+ T cells, (B) mitogen-activated CD8+ T-cells, (C) activated CD8+ T-cells co-cultured with MSCs in standard condition, (D) activated CD8+ T-cells co-cultured with MSCs in transwell (E) Summary of cell cycle analysis with changes percentage of G0-phase and S-phase between activated CD8+ and MLR (n=7, p value=0.014 and 0.006 respectively). Abbreviation: P only = PBMCs only, P+C = PBMCs with ConA, MLR = modified mixed leukocyte reaction

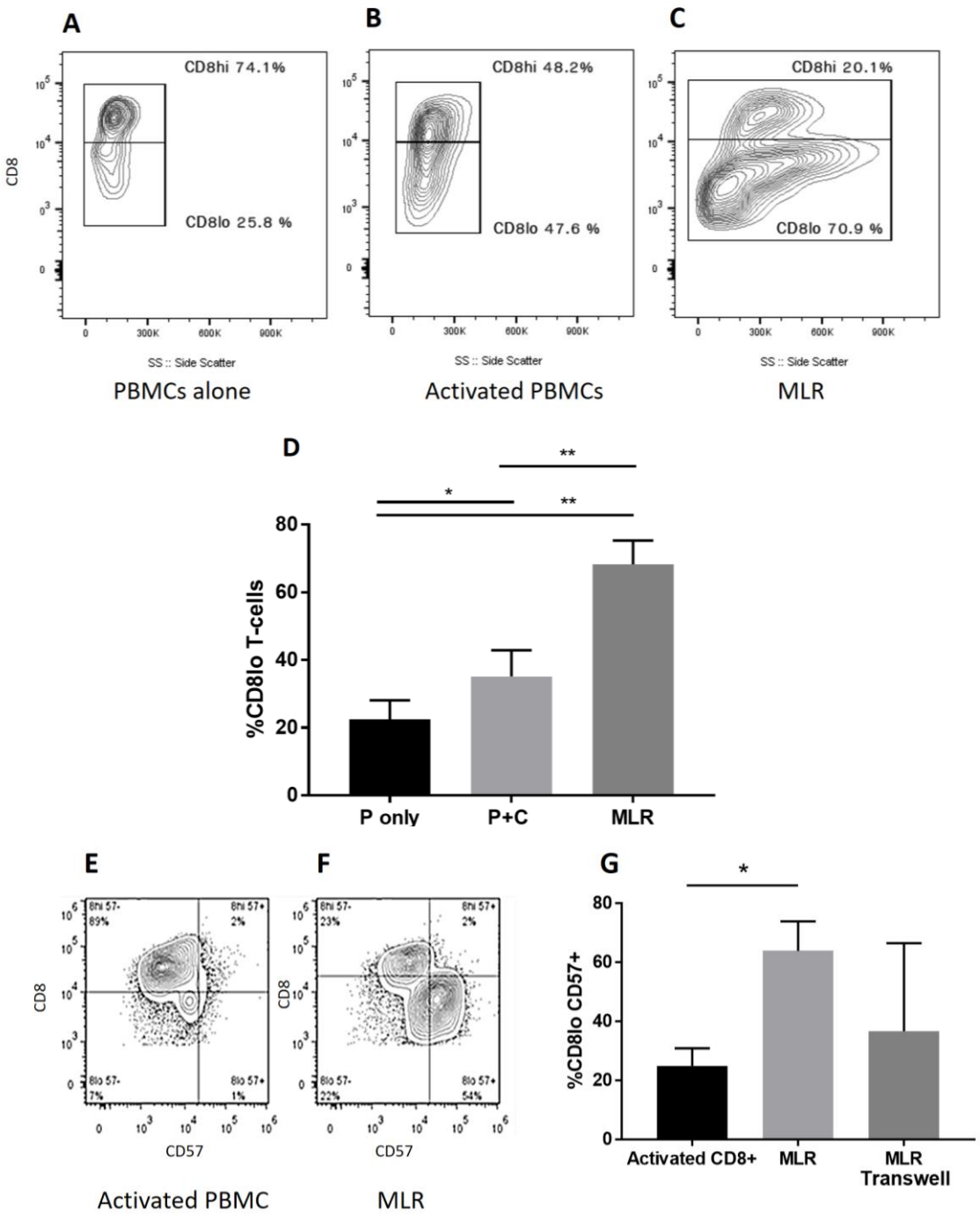


**Figure 3: Feline ASCs downregulate CD8+ T-cell surface expression, increase CD25 and CD57 expression and shift CD8+ T-cells towards a terminally differentiated, effector phenotype** Representative flow-plot on CD8+ T-cell surface expression on (A) non-activated CD8+ T-cells (B) activated CD8+ T-cells (C) activated CD8+ T-cells co-cultured with MSCs. (D) Summary of percentage of CD8lo T-cells (n=5, p-value = 0.007). CD57 expression on (E) activated CD8+ T-cells (F) activated CD8+ T-cells co-cultured with MSCs (G) Summary of percentage of ASCs' induction of CD57+ CD8lo T-cells (n=5, p-value=0.007).

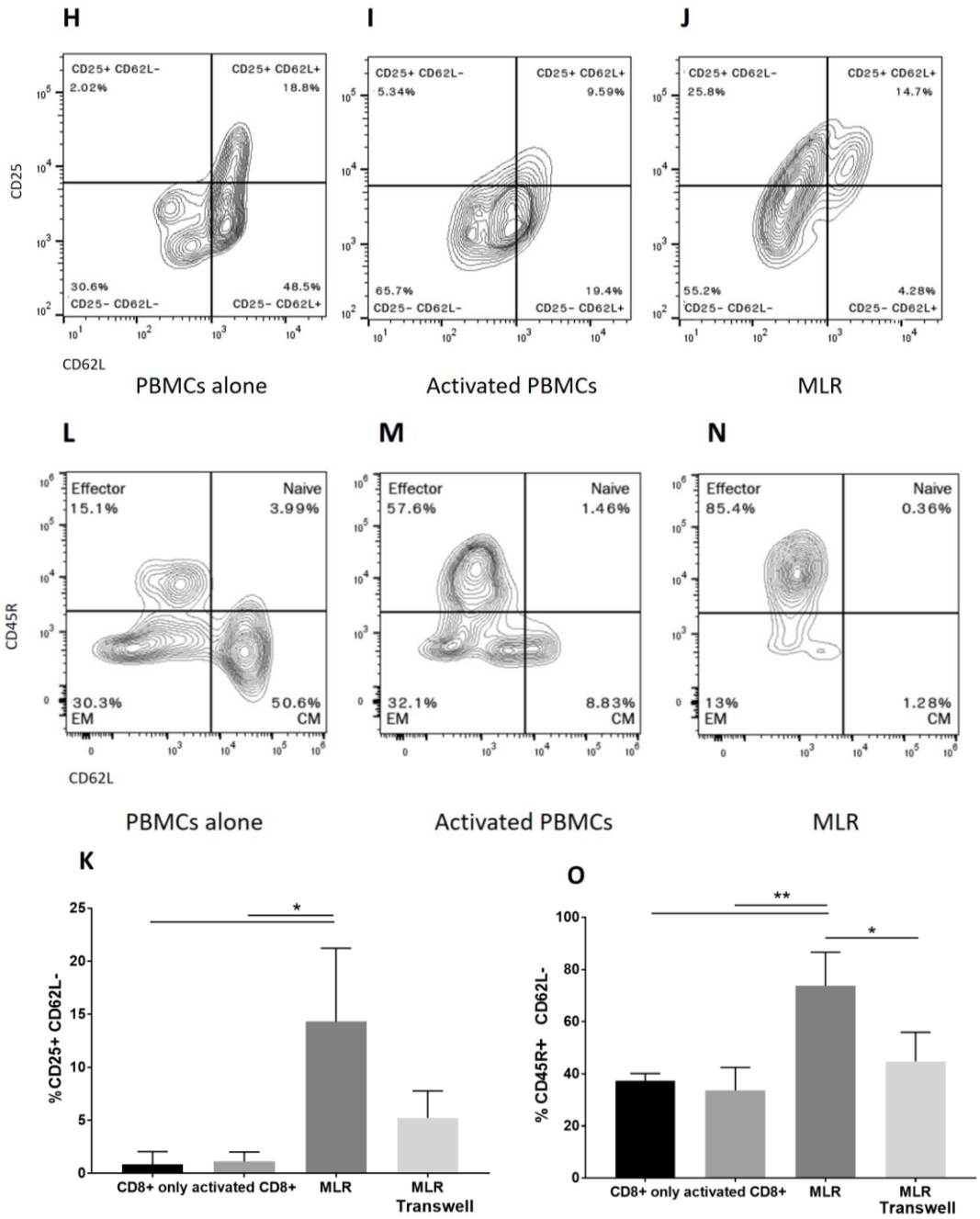


**Figure 3: Feline ASCs downregulate CD8+ T-cell surface expression, increase CD25 and CD57 expression and shift CD8+ T-cells towards a terminally differentiated, effector phenotype** CD25 and CD62L expression on (H) non-activated CD8+ T-cells (I) activated CD8+ T-cells (J) activated CD8+ T-cells co-cultured with MSCs (K) Summary of CD25 and CD62L expression. ASCs increase activation (CD25+) and shedding of L-selectin (CD62L-) on CD8+ T-cells (n=5, p-value=0.028). Expression of CD45R and CD62L to distinguish between effector, effector memory, central memory and naïve CD8+ T-cells in (L) non-activated CD8+ T-cells (M) activated CD8+ T-cells (N) activated CD8+ T-cells co-cultured with MSCs. (O) Summary of increase effector phenotype (CD45R+, CD62L-) on CD8+ T-cells induced by ASCs (n=5, p-value=0.02).

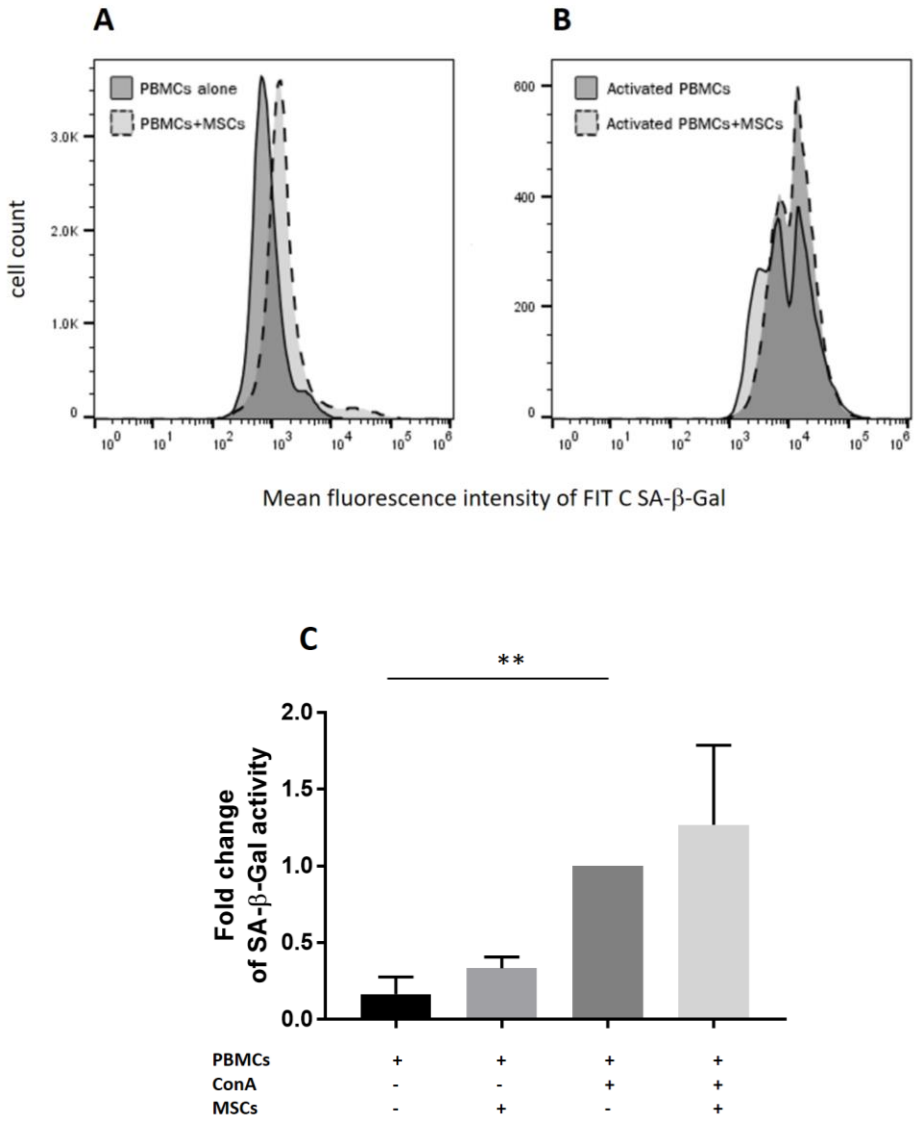


**Figure 4: Feline ASCs do not induce senescence in CD8+ T-cells**. Histogram depicting mean fluorescence intensity for senescence-associated β-galactosidase (SA-β-Gal) positive CD8+ T-cells in (A) PBMCs alone and PBMCs in co-culture with MSCs (B) in activated PBMCs and activated PBMCs in co-culture with MSCs (C) Summary of SA-β-Gal activity with no significant increase between activated PBMCs and MLR and indication of SA-β-Gal activity was mostly from mitogen activation (n=7, p-value=0.007). Fold change calculated based on PBMCs with ConA.

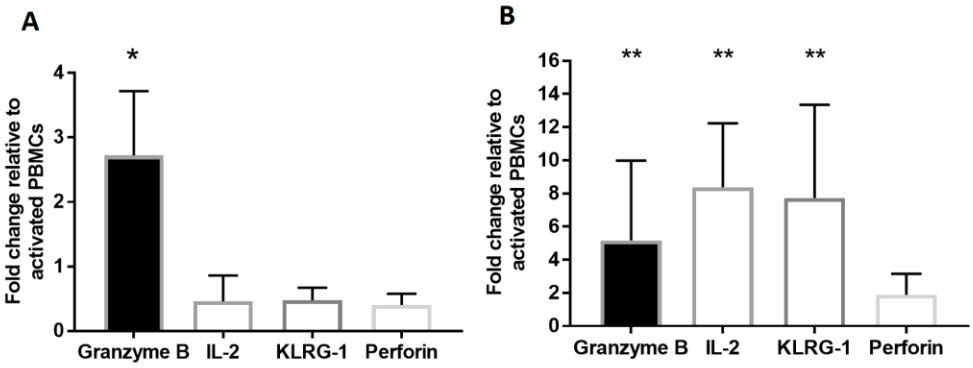


**Figure 5: Feline ASCs upregulate the expression of Granzyme B, IL-2 and KLRG-1** (A) upregulation of granzyme B at 24 hours from PBMCs in co-culture with ASCs in comparison to activated PBMCs alone (n=6, p-value=0.03) (B) upregulation of granzyme B, IL-2 and KLRG-1 at 96 hours from bead-enriched CD8+ T-cells in co-culture with ASCs (n=6, p-value=0.002, 0.03 and 0.002 respectively). Abbreviation: IL-2 – interleukin 2; KLRG-1 – killer cell lectin-like subfamily G1

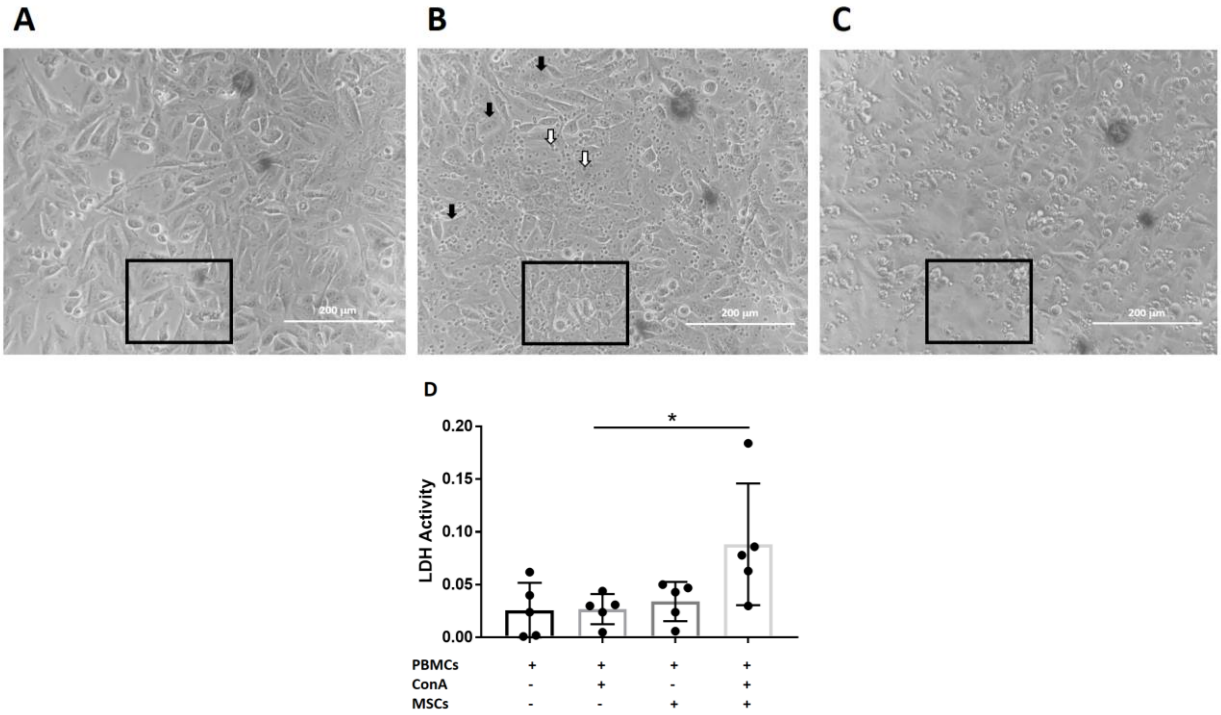


**Figure 6: ASC-primed CD8+ T-cells acquired enhanced cytotoxicity function on target cells** Inverted microscopic images (400X) of (A) CRFK cells alone in culture (B) CRFK cells in culture with bead-enriched CD8+ T-cells from activated PBMCs after 4 hours of incubation (C) CRFK cells in culture with bead-enriched CD8+ T-cells from MLR after 4 hours of incubation. Black arrow head indicates CRFK cells; white arrow head indicates enriched CD8+ T-cells; boxed areas demonstrate loss of monolayer adherence of CRFK cells (D) Summary of increased LDH activity from lysis of target cells by ASC-primed CD8+ T-cells (deducted by spontaneous release of LDH from CRFK cells and enriched CD8+ T-cells alone**)** (n=5, p-value=0.04).

## Results

### Feline ASCs induce cell cycle arrest in CD8+ T-cells in a contact-dependent manner

We previously described that feline ASCs inhibit lymphocyte proliferation, both with or without cell-cell contact. However, the induction of IFN-γ secretion by CD4+ and CD8+ T-cells was enhanced after direct cell contact with ASCs and was mediated through engagement of ICAM-1/LFA-1 ligands [9]. We also determined that the inhibition of lymphocyte proliferation by feline ASCs was due to cell cycle arrest in G0-G1 [3]. Here, we expanded our findings to determine if ASCs induced G0-G1 cell cycle arrest, specifically in CD8+ T-cells, and if this arrest was also associated with direct ASC-CD8+ T-cell contact.

We found that the induction of cell cycle arrest in CD8+ T-cells was more efficient with direct cell-cell contact between feline ASCs and PBMCs (Figure 2A-D) with a significant decrease of CD8+ T- cells in S-phase and increase in G0-G1 phase compared to conA-activated CD8+ T-cells alone (n=7, p= 0.014 and p= 0.006 respectively).

### Feline ASCs downregulate CD8+ T-cell surface expression, increase CD25 and CD57 expression and shift CD8+ T-cells towards a terminally differentiated, effector phenotype

We demonstrated that feline ASCs downregulate CD8+ receptor on the surface of cytotoxic T-cells ($CD8^{lo}$ T-cells) *in vitro* (Figure 3A-D) when compared between MLR and activated PBMCs (p-value = 0.007). Interestingly, the administration of ASCs to cats with FCGS also induced $CD8^{lo}$ T-cells in a cohort of cats that had a positive response to ASC therapy *in vivo* [2]. Here we further characterized the phenotype of CD8+ T-cells after coincubation with ASCs by interrogating the CD8+ T-cells with CD25, CD45R, CD57 and CD62L.

These $CD8^{lo}$ T-cells primed by feline ASCs significantly upregulated surface CD57 expression compared to activated CD8+ T-cells alone (p=0.007) and less so without direct contact (Figure 3E-G). Feline ASCs also upregulated the expression of CD25+ and decreased the expression of CD62L on CD8+ T-cells (Figure 3H-K, p=0.028), possibly indicative of augmented CD8+T-cell activation with CD62L shedding.

Additionally, after interaction with feline ASCs, the activated CD8+ cells shifted to an effector phenotype (CD45R+, CD62L-) compared to activated cells without ASC interaction (p=0.02) (Figure 3L-0). This shift to effector cells is less efficient in the absence of direct cell-cell contact, similar to the expression of CD25 and CD57. These findings collectively suggest that CD8+ T-cells that have interacted with feline ASCs may become senescent cells or terminally-differentiated cytotoxic T-cells.

**Feline ASCs do not induce senescence in CD8+ T-cells**

To date, our data suggest that ASCs induce poorly proliferative CD8+ T-cells that are withdrawn from cell cycle progression, but retain the ability to secrete IFN-γ [9]. These findings could indicate a senescence-associated phenotype. Cellular senescence refers to cells which cease to divide and remain permanently halted in cell cycle progression. We found that mitogen activation induces cell senescence (p=0.007) however feline ASCs did not significantly induce cellular senescence on CD8+ T-cells (Figure 4A-C).

**Feline ASCs upregulate the expression of granzyme B, IL-2 and KLRG-1**

PBMCs which have been primed by ASCs significantly upregulated granzyme B gene expression at 24 hour compared to activated PBMCs alone (p= 0.03, Figure 5A). There was a concurrent slight downregulation for IL-2, KLRG-1 and perforin expression however the changes were not significant. Gene expression at 24 hours could not be performed on enriched CD8+ population due to insufficient amount of isolated RNA.

At 96 hours, activated PBMCs coincubated with feline ASCs upregulated IL-2 (p=0.002) and KLRG-1 (p=0.03). Granzyme B also remains upregulated at 96 hours (p=0.002, Figure 5B).

**ASC-primed CD8+ T-cells acquired enhanced cytotoxicity function on target cells**

Our data suggest that CD8+ T-cells shift to effector cells (CD45R+, CD62L-) with increased Granzyme B expression after incubation with ASCs. We next wanted to determine if the CD8+ T-cells were functionally cytotoxic. To test this, we co-incubated ASC primed CD8+ T cells with virus-infected target cells and measured cellular release of lactate dehydrogenase (LDH). Visually, CD8+ T-cells that were co-incubated with activated ASCs had strong cytopathic effects on virus-infected target cells (Figure 6A-C). These visual data were confirmed by measuring LDH release into the supernatant (released upon cell death). We found that CD8+ T-cells that had been incubated with feline ASCs had enhanced capacity to lyse viral-infected target cells (p=0.04, Figure 6D).

## Discussion

Our current work demonstrates for the first time that feline ASCs can alter the phenotypic and functional characteristics of CD8+ T-cells towards terminally differentiated effector cells. Although proliferative arrest occurred in feline-ASC primed CD8+ T-cells, they remained functionally active and were neither exhausted nor senescent. Activated CD8+ T-cells demonstrated early and sustained upregulation of granzyme B after interaction with feline ASCs with later upregulation of IL-2 and KLRG-1, indicative of enhanced cytotoxicity and a shift towards end-stage differentiation.

Feline ASCs have been evaluated for their therapeutic effects in various experimental models and clinical trials relating to inflammatory and immune-dysregulated diseases [1-4, 15, 16]. In feline patients with chronic gingivostomatitis (FCGS), administration of ASCs resulted in decreased CD8+ T-cells and the downregulation of CD8 receptor expression, both corresponding to a positive response to ASC therapy [2]. CD8+ T-cells play an important role in the pathophysiology of FCGS, especially with a potential underlying cause of viral infection, particularly Feline Calicivirus (FCV) infection [17].

In this study, we focused on ASC regulation of feline CD8+ T cells as in our clinical trial in cats with FCGS, CD8+ T cells are a clear biomarker associated with infection and phenotype change is associated with disease resolution in some cats. Published in vitro data on CD8+ T cells in mice and people primarily suggest that MSCs dampen T cell proliferation and shift CD8+ T-cells towards a suppressive or regulatory phenotype [18-20], here we have demonstrated feline ASCs augment CD8+ T-cell effector function *in vitro*.

When CD8+ T-cells expand and differentiate during activation, most cells will terminally differentiate into end-stage effectors that have a shortened lifespan and die while a smaller subset of cells differentiates into memory cell precursors [21]. Cytotoxic CD8+ T-cells are a crucial component of the adaptive arm of the immune system. One primary role is to combat intracellular pathogens, utilizing granzyme and perforin stored in their cytotoxic granules to kill virally infected cells. Granzyme B, among other members in the granzyme family, has the most potent apoptotic function [22].

Our study showed that feline ASCs induce cell cycle arrest on CD8+ T-cells in a contact-dependent manner. Poorly proliferative CD8+ T-cells can be the result of a shift to terminal differentiation, functional exhaustion or cellular senescence [23-25]. ASCs do not induce an exhausted phenotype as ASC-primed CD8+ T-cells retain their cytokine-secreting ability including IFN-γ [26] and IL-2 expression.

Senescent and terminally differentiated CD8+ T-cells have several overlapping cellular features. Replicative cellular senescence is commonly associated with aging and is explained by telomere shortening after cells have undergone numerous replicative cycles [24]. Premature senescence, which is telomere-independent, can be induced by cellular stress, DNA damage or T regulatory cells [27, 28]. Feline ASCs induced CD25 (IL2 receptor) upregulation. IL-2 plays a pivotal role in the survival, clonal expansion and promoting CD8+ T-cells toward effector differentiation [29]. Moreover, feline ASCs also induced shedding of L-selectin (CD62L) a lymph node homing receptor, and upregulated CD45R expression, indicative of an effector phenotype. Additionally, cytokine milieu also affects effector cell function and feline ASCs secrete several

immunomodulating cytokines and also augment IFN-γ secretion from T-lymphocytes [26], possibly indicative of their drive towards short-lived cytotoxic CD8+ T-cells [30].

We also determined that these CD8+ T-cells were not senescent through the detection of intracytoplasmic senescence-associated β-galactosidase enzyme in CD8+ T-cells. Mitogen stimulation did induce cellular senescence however ASCs neither induced senescence nor rescued PBMCs from activation/ replication-induced senescence. Given that the CD8+ T-cells were not senescent or exhausted and their phenotype was most like effector cells, we confirmed these findings using gene expression and a functional assay.

Killer cell lectin-like receptor subfamily G1 (KLRG-1) is an inhibitory marker expressed on NK cells and T-cells, predominantly associated with CD8+ effector cells retaining cytokine production capacity, but limited proliferative ability while approaching the end of T-cell differentiation. [31, 32]. Our data showed increased expression of granzyme B and KLRG-1 supportive of CD8+ T-cell differentiation. Similar to our findings, in humans, the KLRG-1+ population of CD8+ T-cells has increased granzyme B expression [33]. These findings were further confirmed by increased surface expression of CD57, an NK cell and T-cell marker most prominently expressed in terminally differentiated effector T-cells and mature cytotoxic NK cells [34].

Lastly, we reported enhanced cytotoxic functionality of feline ASC-primed CD8+ T-cells through an LDH-release of virally-infected target cells. We utilized Crandell-Reese feline kidney (CRFK) cells, an immortalized allogeneic mesenchymal cell line [35], as the cell target to examine the effects of CD8+ T-cell-mediated cytotoxicity. Destruction of target cells through TCR-MHC-class I and/or Fas-ligand interaction [36] between activated feline CD8+T-cells and

CRFK cells was measured through lactate dehydrogenase (LDH) release. LDH is present in all mammalian cells and the release in culture supernatant was quantified in comparison to target cells (CRFK) alone and effector cell (enriched CD8+ T-cells) alone with maximal lysis control after 4 hours of co-incubation. Our cytotoxicity assay indicated an approximately 61% increase of CRFK cell death from feline ASCs primed activated CD8+ T-cells. We also noted that the FCV infection on target cells was not necessary for cell lysis (data not shown).

## Conclusion

Our findings collectively suggest that feline ASCs 1) alter CD8+ T-cells towards terminally differentiated, proinflammatory (IFNγ, CD25 (IL2 receptor), IL2) effector phenotype ($CD8^{lo}$, CD62L-, CD45R+) with limited proliferative capacity, and 2) enhance their cytotoxic potential through granzyme B upregulation. These data mimic some of our findings in vivo after ASC administration to cats with FCGS, a disease characterized by a CD8+ T-cell proinflammatory phenotype. These cytotoxic CD8+ T-cells could aid in disease cure in cases caused by an underlying, unresolved viral infection.

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# Chapter 4

## Immunological effects of mesenchymal stem cell therapy on T-lymphocyte subsets in cats with chronic gingivostomatitis

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**Abstract**

Feline chronic gingivostomatitis (FCGS) is an inflammatory oral disease with CD8+ T cell mediated component in the periphery and possibly associated with chronic viral infection. Adipose-derived mesenchymal stem cells (ASCs) can be effectively used for the treatment of refractory FCGS. Our study investigated the phenotypical changes on T-cells in FCGS patients after ASC therapy and showed that feline ASCs may help resolve FCGS by decreasing T-cell activation. FCGS patients had decreased central memory CD8+ T-cell (TCM) in lymphoid tissue and with lowered CD8+ T-regulatory (T-regs) cell population in circulation and CD4+ T-regs in lymph nodes. These findings may help define other immune-mediated disease targets that could benefit from ASC therapy.

**Keywords:** feline chronic gingivostomatitis, mesenchymal stem cell, CD8+ T-cells

### Introduction

Feline chronic gingivostomatitis (FCGS) is a severe, debilitating inflammatory oral disease. FCGS is characterized by the persistent, erosive inflammation which extends beyond mucogingival junction to the caudal oral mucosal tissue [1], causing extreme discomfort, halitosis, anorexia and weight loss.

Although the causes of FCGS are deemed multifactorial and still largely undetermined, it is generally accepted that FCGS resulted from the host's immune dysregulation secondary to pathogen, particular viruses [2]. The prevalence of FCGS also increases in households with multiple cohabiting cats, further suggestive of its infectious origin [3]. Aside from possible genetic predisposition and stresses, several viruses have been raised as potential causes, including feline leukemia virus (FeLV), feline immunodeficiency virus (FIV), feline herpesvirus 1 (FHV-1) and feline calicivirus (FCV). A recent transcriptomic study showed that FCV had the strongest association to FCGS pathogenesis [4].

Current standard management for FCGS involves medical management with antibiotic therapy, and immunosuppressive drugs or surgical intervention with a full-mouth tooth extraction [1] with varying degree of responses. FCGS cats that do not respond to conventional therapy are often chosen to be humanely euthanized due to poor quality of life [5].

FCGS cats typically have systemically elevated CD8+ T-cells with activated phenotype (CD25+, CD62L-) with increased effector memory T-cells in circulation [6], indicating a sustained inflammation and a protracted antiviral response. Adipose-derived mesenchymal stem cells (ASC) administration has been utilized successfully as an alternative option for the treatment of refractory FCGS [7, 8] due to their capability of modulating the immune response [9]. Our previous study has shown that infused ASCs trafficked to the inflamed oral cavity in FCGS cats [7]. ASCs are short-lived systemically after administration and the majority of infused ASCs were entrapped in the pulmonary capillaries due to their large size [10], hence the long-lasting immunomodulatory effects of ASCs and their alterations on immune cell subsets in diseased animals remain to be explored.

Our study aimed to investigate the phenotypical changes on T-cells in FCGS animals after systemic administration of ASCs, both in the periphery and in the lymphoid tissue. This may shed light on this subpopulation of immune cells affected by ASCs and potential implementation of ASC therapy towards similar immune-mediated disease models in other small animals.

## Materials and Methods

### Animal use and ethical consent

Animals included were enrolled from two studies. Both studies were reviewed and approved by UC Davis Institutional Animal Care and Use Committee (AICUC) and by the Clinical Trial Review Board (CTRB) at the University of California, Davis. In addition, a written informed consent was obtained from all owners, comprising of fourteen client-owned cats with refractory FCGS with no other co-morbidities.

Blood and mandibular lymph nodes were obtained at time 0, prior to ASC administration, and at 6 months after ASC administration (study end date). Paired blood and mandibular lymph nodes were also collected from 5 control cats (both genders, 2-15 years of age) that were euthanized for reasons not related to this study. These cats had no evidence of systemic inflammation, infection or neoplasia.

The mandibular lymph nodes were collected with the cat under general anesthesia and the area of the ventral mandibles surgically prepared for aseptic surgery. Following a skin and subcutaneous incisions, the lymph nodes were isolated and removed followed by a routine closure.

**Lymph node sample processing**

Lymph nodes obtained from healthy and FCGS patients were dissected in biosafety cabinet to small 1x1 mm pieces with a scalpel. The lymph node tissues were pressed through a 70 μm pore cell strainer, pelleted at 400g for 5 minutes and washed twice in DPBS before resuspending in feline flow buffer (DPBS –Ca –Mg + 2% FBS (Atlanta Biologics), 2 mM EDTA, in DPBS) prior to cell labelling.

**Cell phenotyping**

Lymphocyte phenotyping was performed with flow cytometry on peripheral blood mononuclear cells (PBMCs) separated through Ficoll gradient or on cells obtained from lymph nodes.

**Table 1:** Antibody utilized for cell phenotyping on flow cytometry

| Antigen | Samples | Clone (source) | Volume / Dilution |
|---|---|---|---|
| CD4 | LN | Mouse FE1.7B12 Unconjugated (LABL) | 25 μl |
| | PBMCs | Mouse 3-4F4-PE (Southern Biotech) | 1 μl |
| CD5 | PBMCs | Mouse F43-FIT C (Southern Biotech) | 1 μl |
| CD8 | LN | Mouse FE1.10E9 Unconjugated (LABL) | 25 μl |
| | PBMCs | Mouse FE1.10E9-PE (LABL) | 1 μl |
| CD45R | PBMCs / LN | Mouse RA3-6B2(B220) APC-Cy7 (Biolegend) | 1.25 μl |
| CD62L | PBMCs / LN | Mouse LAM1-116-APC (MyBioSource) | 2.5 μl |
| FoxP3 | PBMCs / LN | Mouse FJK-16Ss-APC (eBioscience) | 2.5 μl |
| T-cell activation marker | PBMCs | Activated T Lymphocyte Cocktail (BD Pharmingen) | 20 μl |
| Anti-mouse Ig (H+L), F(b)'2 | | Donkey α mouse-PE (Jackson Immuno) | 1:50 |
| Streptavidin- PE | | (BD Biosciences) | 1:2,000 |

LABL: Leukocyte Antigen Biology Lab, UC Davis, LN: lymph nodes

Antibody clones used for phenotyping is listed in Table 1. Blood was ficolled to separate PBMCs according to a modified method [11]. In brief, blood was diluted with 2 volumes of warm Tyrodes HEPES buffer (12 mM NaHCO3, 138 mM NaCl, 2.9 mM KCl, 10 mM HEPES, 2.5 mM EDTA, pH 7.2). Diluted blood was layered on top of a discontinuous gradient (Ficoll-Paque Plus diluted 6:1 with sterile water, on top of Histopaque-1.119) (Sigma, St. Louis, MO). Samples were centrifuged at 400 g for 20 min with zero deceleration. The PBMC layer was transferred into a fresh tube and washed twice with DPBS before resuspending in flow buffer. Cell surface markers were labeled by incubating with primary antibody for 30 min at room temperature and washed twice in flow buffer. Samples labeled with an unconjugated primary antibody were then incubated with a secondary antibody diluted in 100 µL flow buffer for 20 min at room temperature.

Samples for intracellular FoxP3 labeling were further processed using a FoxP3 fixation/permeabilization kit (eBioscience), following the manufacturer's instructions. They were then washed twice in flow buffer containing 0.1% saponin (Perm/Wash buffer) and blocked with 2% rat serum in Perm/Wash for 15 min before the FoxP3 antibody was added. Cells were incubated at 4°C for 30 min, then washed twice with Perm/Wash, before suspension in flow buffer.

All labeled cells were analyzed on FC500 flow cytometer using Cytomics CXP software (Beckman Coulter) and FlowJo software (Ashland, OR). The gating strategies for cell phenotyping is illustrated in Figure 1.

**Statistical Analysis**

Normal distribution of the data was tested using the Shapiro-Wilk test. A paired t-test (normally distributed data) or Mann–Whitney U test (non-normally distributed data) was used to determine differences in between two groups in data set. Difference between 3 groups was determined using Kruskal-Wallis test with Dunn's multiple comparison post-test. A commercially available statistical software was used for all statistical analyses (GraphPad InStat version 3.06 for Windows; GraphPad Prism, San Diego, CA). A P value of <0.05 was considered statistically significant.

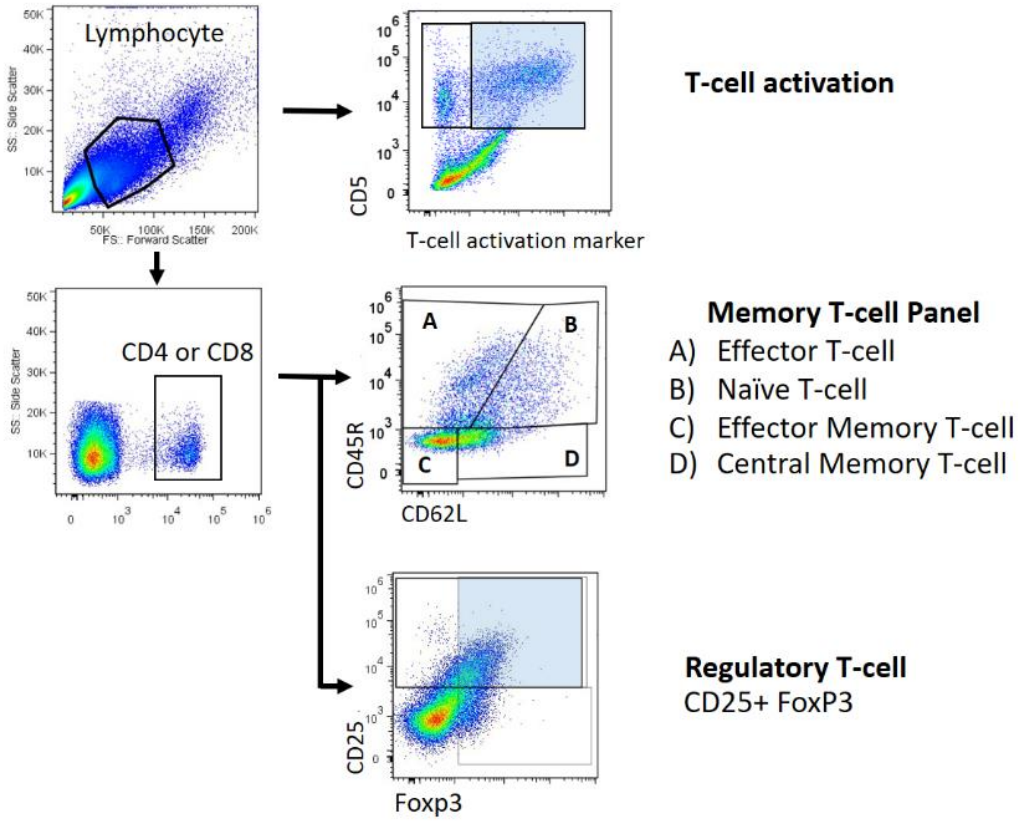



**Figure 1:** Gating strategy for T-cell phenotyping

## Results

### Cat

Of the 14 refractory FCGS cats were enrolled into the study, 12 completed the study and 2 were lost to follow-up. 7 patients (58.33%) demonstrated clinical improvement after ASC therapy and 5 patients had no improvement. Lymph nodes were obtained from 8 of the FCGS patients (4 patients with clinical improvement, 3 patients without improvement and 1 patient lost to follow-up). Signalment of study population, cell source and clinical response are listed in Table 2.

**Table 2:** Age, gender and outcome of ASC therapy in enrolled FCGS patients

| Animal No. | Age (years) | Gender | ASC Source | Clinical outcome of treatment |
|---|---|---|---|---|
| **1** | 9 | Fs | Autologous | Substantial improvement |
| **2** | 7 | Fs | Autologous | Substantial improvement |
| **3** | 1 | Mc | Autologous | Substantial improvement |
| **4** | 3 | Mc | Autologous | No improvement |
| **5** | 9.5 | Mc | Autologous | Clinical Cure |
| **6** | 11 | Mc | Allogeneic | Minimal improvement |
| **7** | 7 | Fs | Autologous | Lost to follow-up |
| **8** | 2.5 | Mc | Allogeneic | No improvement |
| **9** | 2 | Mc | Allogeneic | Lost to follow-up |
| **10** | 12 | Mc | Allogeneic | Substantial improvement |
| **11** | 2.5 | Mc | Allogeneic | Substantial improvement |
| **12** | 5 | Fs | Allogeneic | No improvement |
| **13** | 6.5 | Mc | Allogeneic | No improvement |
| **14** | 8 | Mc | Allogeneic | No improvement |

Fs: spayed female, Mc: castrated male

### ASC therapy decreased T-cell activation in the blood of FCGS patients, decreased central memory CD8+ T-cell in lymph nodes and shifted phenotype to effector memory T-cell

Feline patients suffering from FCGS had similar baseline level of activated T-cells to healthy control and ASC administration significantly lower T-cell activation compared to prior to treatment (Figure 2A-D, p-value = 0.07).

Baseline level of central memory (CD45R-, CD62L+, $T_{CM}$) CD8+ T-cells in the lymph nodes of FCGS patients were significantly higher than healthy controls (Figure 3A-E, 3D: p value = 0.006). ASC therapy appeared to help normalize the increased population of central memory CD8+ T-cells, creating a shift of CD8+ T-cell population to effector memory (CD45R-, CD62L- , $T_{EM}$) T-cells. The level of $T_{EM}$ in FCGS patients was significantly higher (Figure 3H; p value = 0.002) while $T_{CM}$ was lowered in the blood (Figure 3G; p value = 0.004) compared to healthy controls. However, no remarkable changes concerning effector or memory phenotype was observed in the periphery.

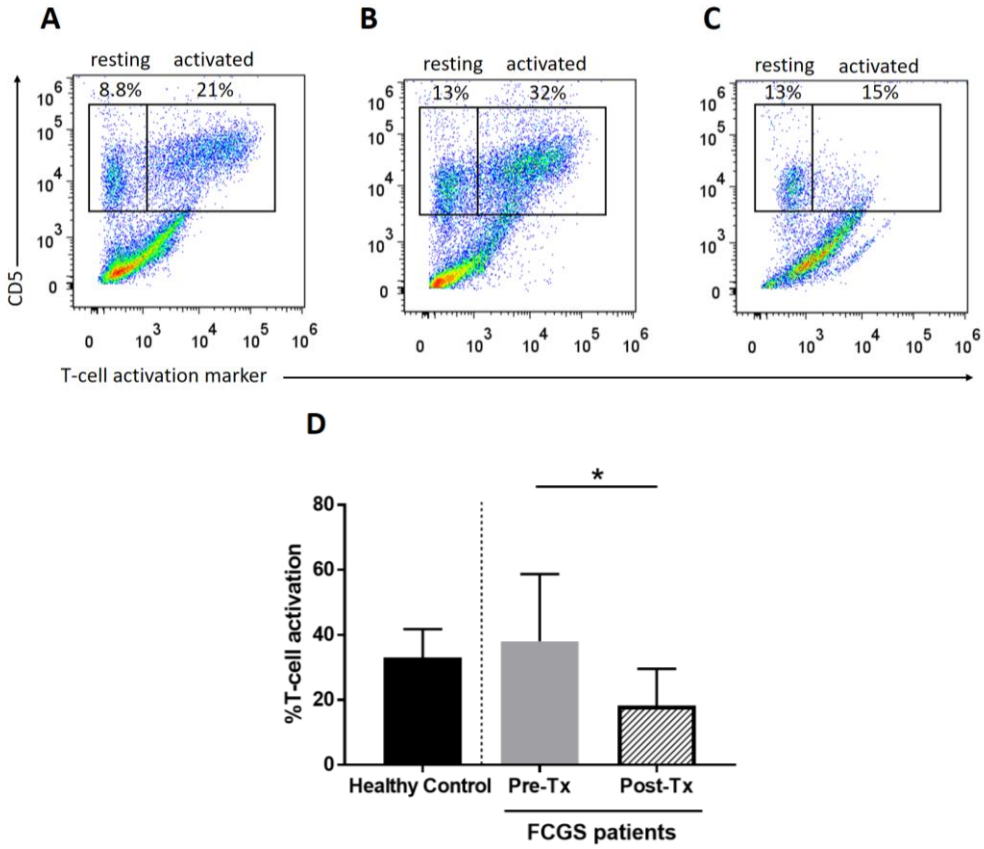
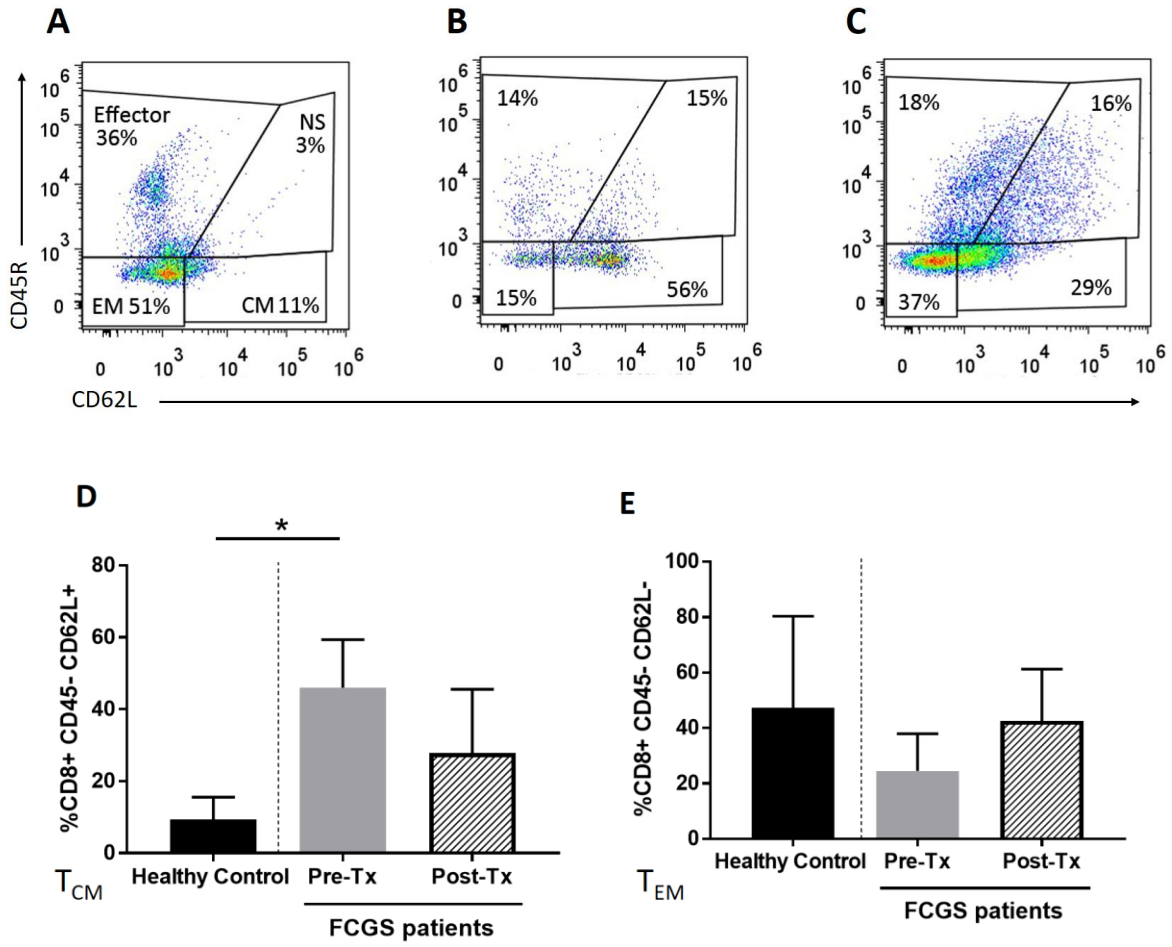



**Figure 2:** Representative flow cytometry plots on activated T-cells prior to and after ASC therapy (A) healthy control (B) FCGS patients prior to ASC therapy (C) FCGS patients after ASC therapy (D) Summary graphs on activated T-cells prior to and after ASC therapy in lymph nodes.

**FCGS patients undergone ASC therapy have decreased CD8+ T-regs (CD25+ FoxP3+) in peripheral blood and CD4+ T-regs in lymph nodes**

The regulatory subset (CD25+ FoxP3+) was affected by ASC administration both in peripheral blood and in lymph nodes of FCGS patients. The CD8+ T-regs of FCGS patients were elevated compared to healthy control patients and remained high after therapy (Figure 4J). However, the CD8+ T-reg level in the blood greatly decreased after treatment (Figure 4E, p value = 0.007). ASC therapy also significantly reduced the percentage of the CD4+ T-regs population in lymph nodes (Figure 4D, p-value = 0.0047) with no obvious alterations in the blood.



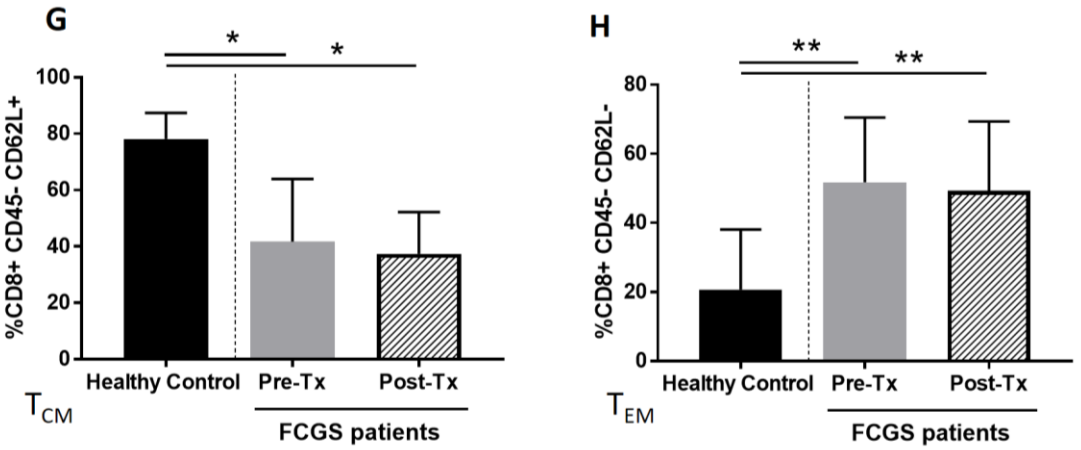
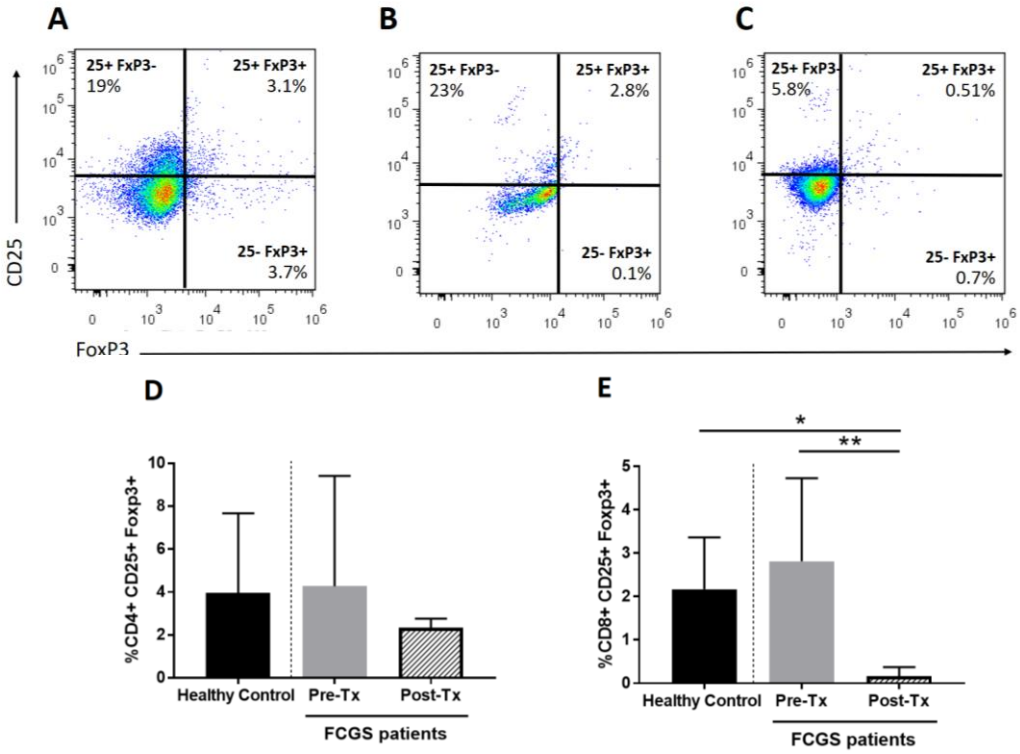



**Figure 3:** Representative flow cytometry plot of on memory T-cell panels in lymph nodes of FCGS patients T-cells prior to and after ASC therapy (A) healthy control (B) FCGS patients prior to ASC therapy (C) FCGS patients after ASC therapy (D) Summary graphs on (D) central memory CD8+ T-cells and (E) effector memory in the lymph nodes and (F and G) in the blood prior to and after ASC therapy



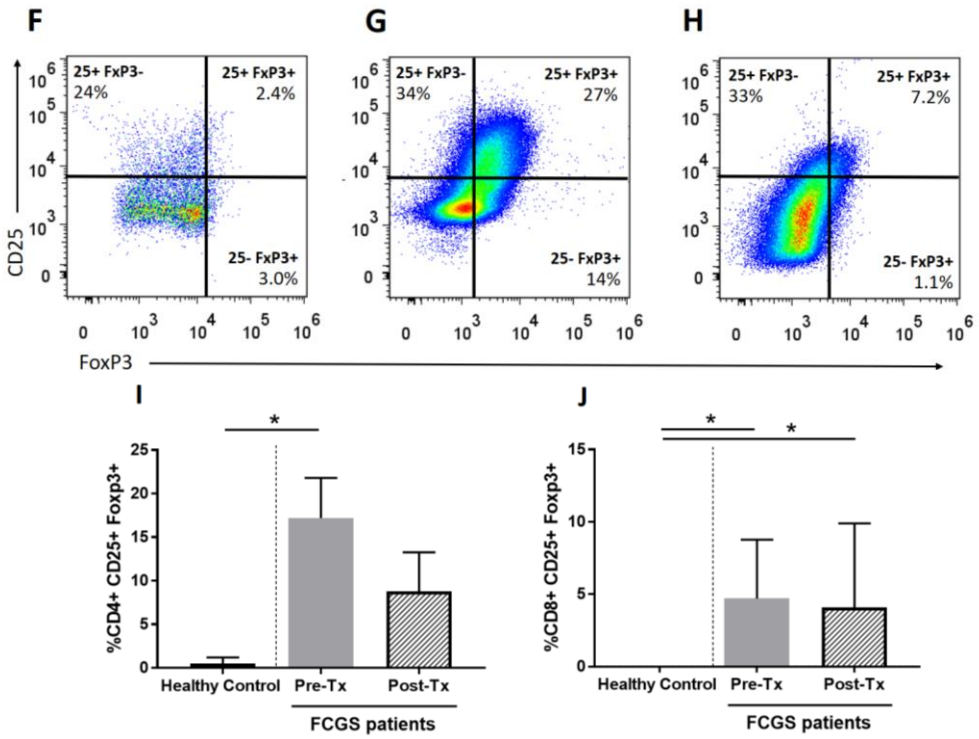



**Figure 4:** Representative flow cytometry plot of on regulatory CD8+ T-cells (CD25+ FoxP3+) in peripheral blood of FCGS patients T-cells prior to and after ASC therapy (A) healthy control (B) FCGS patients prior to ASC therapy (C) FCGS patients after ASC therapy (D, E) Summary graph of CD4+ and CD8+ T-regs in peripheral blood. Representative flow cytometry plot of on regulatory CD8+ T-cells (CD25+ FoxP3+) in lymph nodes of FCGS patients T-cells prior to and after ASC therapy (F) healthy control (G) FCGS patients prior to ASC therapy (H) FCGS patients after ASC therapy (I, J) Summary graph of CD4+ and CD8+ T-regs in lymph nodes.

## Discussion

We explored, for the first time, the outcome of phenotypical alterations in T-cell populations following ASC therapy in FCGS patients. The positive response rate of this study at approximately 58%, either resulting in clinical improvement or complete cure from refractory FCGS after ASC administration, was similar to the previously published clinical trial using allogeneic mesenchymal stem cells [7].

With FCGS being characterized by sustained inflammation, elevated white cell counts and increased levels of circulating CD8+ T-cells [6], it is not surprising that ASC therapy decreased T-cell activation. T-cells could only function to clonally expand and proliferate upon activation as we also previously found that feline ASCs could inhibit T-cell proliferation both through secreted mediators and cell-cell contact [12].

A previous study demonstrated that FCGS patients have increased $T_{EM}$ and decreased $T_{CM}$ in peripheral blood [6] and our study also found the same trend, however, ASC therapy did not significantly impact these populations; but, the observed changes were within the lymphoid tissue. Effector memory ($T_{EM}$) CD8+ T-cells are hypothesized to be adept at responding to infections in peripheral organs due to their cytotoxicity and ability to localize to tissues [13], compared to their central memory ($T_{CM}$) counterparts that are more specialized to handle systemic infection. In chronic viral infection, the population of $T_{CM}$ and aged/late memory cells are expected to proliferate in response and produce IL-2 to enhance protection [14]. Hence, in our study, the percentage of $T_{CM}$ population was significantly increase in the lymph nodes in FCGS patients compared to healthy control and was normalized following ASC therapy, possibly indicating a resolution of sustained antiviral response.

Regulatory compartment of T-cells is indispensable for the mitigation of overactive inflammation response. Our study showed that percentage of the CD8+ T-regs was lowered in circulation and CD4+ T-regs was also decreased in lymphoid tissue following ASC administration. In infectious conditions, CD8+ T-regs help limit immune response against pathogens to prevent excessive tissue damage [15]. However, with certain virus infections, CD8+ T-regs reduce the population of virus-specific effector T lymphocytes [16] with their

ability to suppress the proliferation of both CD4+ or CD8+ effector cells [17] which may be counterproductive when managing FCGS with persistent viral infections. There was no corresponding shift between periphery and lymphoid trafficking of CD4+/CD8+ T-regs, indicating that it is a true decrease in percentage and not a change in lymph node homing behavior of T-regs by ASC therapy.

It is difficult to define whether these systemic changes in T-cell subsets were altered directly by ASCs or whether the downstream effects of resolved inflammation or elimination of chronic viral infection as we recently found that feline ASCs could enhance CD8+ T-cell's cytolytic function *in vitro* [article under review]. As FCGS was also appeared to be partially B-cell mediated [6], future research on changes of other immune cell subsets and/or viral status of patients after ASC therapy could provide further insights into the process of disease cure.

## Conclusion

Our study demonstrated that feline ASC therapy in FCGS patients resulted in decreased T-cell activation, lowered CD8+ $T_{CM}$ in secondary lymphoid tissue as well as reduced percentage of regulatory T-cell population. Further study to determine if this changes correlate to alteration of other immune cells and the viral status of FCGS patients and would be beneficial to the understanding of how ASCs helped resolve refractory FCGS.

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