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Mechanisms of lymphocyte loss in SARS coronavirus infection

Key Messages

1. Human lymphocytes and monocytes are not permissive to productive SARS coronavirus (SARS-CoV) infection in vitro.
2. Challenge of lymphocytes and monocytes with infectious SARS-CoV, inactivated virions, and receptor-binding fragment of spike protein does not trigger apoptosis.
3. Direct infection/interaction between viruses and lymphocytes/monocytes is unlikely to be the cause of lymphopaenia in SARS patients.
4. Lymphopaenia in SARS patients is likely to result from indirect mechanisms secondary to the viral infection.

Introduction

SARS is an emerging infection caused by the SARS coronavirus (SARS-CoV), characterised by fever, non-productive cough, dyspnoea, and in severe cases, respiratory failure. In addition, lymphopaenia has been reported in 68 to 98% of SARS patients.[1] To date it is not known whether the decrease in lymphocyte count is the result of SARS-CoV directly infecting the cell or mediated via indirect mechanisms.

Viral infections often result in death of host cells, perhaps as a host defence mechanism to reduce virion production and limit spread, but may also be exploited by the virus for dissemination. The best studied mechanism of virus-induced host cell death is apoptosis, or programmed cell death, involving chromatin condensation, DNA fragmentation, and formation of apoptotic bodies. Apoptosis has been observed in monocytes/macrophages infected with human coronavirus strain 229E.[2] A few studies have attempted to address the possible mechanisms of lymphocyte loss in SARS patients, but the findings have not yielded conclusions.[3,4] This study examined the possible mechanisms leading to lymphopaenia in SARS-CoV infection—with particular focus on the permissiveness of lymphocytes to viral replication, and possible lymphocyte apoptosis following virus–host cell interactions.

Methods

This study was conducted from February 2005 to February 2007.

Cells

Four T lymphoid cell lines (Jurkat, HSB2, MOLT3, and SupT1) together with peripheral blood mononuclear cells (PBMC) collected from healthy donors were used in parallel for all infection/treatment experiments. Prior to inoculation, PBMC were thawed and separated into adherent (monocytes) and non-adherent (lymphocytes) fractions, by resting them overnight at 37ºC.

SARS-CoV isolates

Three SARS-CoV isolates were used, including the CUHK-Su10 isolated from the hotel Metropole cluster in Hong Kong, which was closely related to the prototype Urbani from Vietnam and the TOR2 strain from Canada. The other strain CUHK-W1 is phylogenetically distinct from CUHK-Su10. The third was the CUHK-LC2 which has a large 386-bp deletion. This strain emerged towards the end of the outbreak in Hong Kong.

Permissiveness of lymphocytes to productive SARS-CoV infection

Infection with SARS-CoV

T lymphoid cell lines (Jurkat, HSB2, MOLT3, and SupT1), and healthy donor–derived PBMC were resuspended to $1x10^7$ cells/mL, and inoculated with the three SARS-CoV strains at multiplicity of infection (MOI) of 0.1, 1, and 10. Experiments were performed in triplicate and harvested for viral RNA quantitation at day-2, -4, -6, and -8 post-infection. Cytopathic effects were recorded before each harvest. Cells collected at day 8 were used for viral protein detection. Vero cells were used as positive controls. Non-infected Vero cell culture supernatant was used for mock infection.

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Lymphocyte and monocyte co-culture
Entire PBMC preparations were also used without prior separation into adherent and non-adherent fractions. This allowed co-culture of lymphocytes and monocytes with SARS-CoV, where the interaction between these cell types may be important for infection and/or apoptosis.

Antibody-dependent effects
To investigate for the possible antibody-dependent effects on infection and apoptosis, the virus preparations were treated with convalescent plasma for 1 hour at 37ºC, to allow virus-antibody complex formation prior to inoculation to PBMC.

Intracellular viral protein detection
It was done by immunofluorescence assay based on SARS-CoV nucleocapsid protein-specific antibody. Infected/treated cells were harvested for staining at the end of incubation (day 8).

Intracellular viral RNA quantification
This involved total RNA extraction from cell pellets for measurement of intracellular viral RNA level. First-strand cDNA was synthesised by SuperScript III reverse transcriptase (Invitrogen, CA). SARS-CoV RNA strand-specific primer was used (nsarsF 5'-CAGCCCCAGATGGTACTTCT-3' is minus strand-specific, targeting for replicate intermediate RNA; or nsarsR 5'-AGCGCCGTAGGGAAGTG-3' is plus strand-specific, targeting for genomic RNA). First-strand cDNA was amplified using the Power SYBR Green kit (Applied Biosystems, CA), with nsarsF and nsarsR primers. Samples were tested in triplicate. The GAPDH mRNA level was used as a reference to normalise for the variations in amounts of cells harvested.

Apoptosis of lymphocytes
Lymphocyte-derived cell lines and PBMC fractions were examined for apoptosis following treatment with:
(1) Live SARS-CoV—the three strains of SARS-CoV were inoculated respectively at MOI of 0.1, 1.0 and 10, as described above.
(2) Inactivated SARS-CoV—this was obtained by firstly freezing-and-thawing of a SARS-CoV–infected Vero cell suspension, followed by virus inactivation at 60ºC for 30 min. Cell debris was removed by centrifugation at 1300 rpm. Absence of live virus in these preparations was confirmed by inoculation onto Vero cell monolayers and incubation for 10 days.
(3) Recombinant SARS-CoV spike protein containing the receptor-binding fragment.

A DNA fragment encoding the receptor-binding domain, amino acid residues 318-510, of the SARS-CoV spike protein was synthesised in a yeast expression system. A synthetic pre-S portion of hepatitis B virus surface protein prepared in the same way was used as control. After receiving the above treatments, the cell cultures were incubated at 37°C for 8 days. The presence of apoptosis was detected by multiple methods:

(1) DNA fragmentation—cells were harvested daily until day 8. Cells were heated at 95°C for 15 min to kill any virus present, then lysed and treated with proteinase K. The extracted DNA was analysed by agarose gel electrophoresis.
(2) TUNEL assay—cells were harvested daily to detect apoptotic nuclei using the DeadEnd Colormetric TUNEL System (Promega, WI).
(3) Annexin V staining—cells were harvested daily for staining using the Annexin V-FITC apoptosis detection kit (BD Biosciences, NJ). The results were observed with fluorescent microscopy. Cells treated with non-infectious preparations were also examined by flow cytometry (Beckman Coulter, CA).

Results

Cytopathic effects and intracellular viral protein production
No definite cytopathic effects were detected in the lymphocyte cell lines and PBMC after inoculation with CUHK-W1, Su10, and LC2. A small proportion (<5%) of the PBMC showed dull green-to-yellow signals, which could represent non-specificity or a low level of viral protein. Similarly, a small proportion (also <5%) of the PBMC incubated together with convalescent plasma also showed dull green-to-yellow signals. The signals observed for the PBMC plus convalescent plasma was stronger than those from PBMC alone (data not shown).

Intracellular plus- and minus-strand viral RNA level
The results of SARS-CoV plus-strand (genomic) and minus-strand (replicate intermediate) real-time polymerase chain reaction are shown in Figure 1. High levels of plus-strand (normalised: 2547-3869 copies/μL) and minus-strand (3158-4369 copies/μL) viral RNA were found at day-2 and -4 post-inoculation for Vero cells. For lymphocyte cell lines, only Jurkat cells showed a detectable level of plus-strand viral RNA at day 2 (36 copies/μL for MOI=10, 11 copies/μL for MOI=1.0); and minus-strand viral RNA was only detected in Jurkat cells at day-2 post-inoculation at MOI of 10. For PBMC, plus-strand viral RNA was detected at low levels (2-62 copies/μL) from the PBMC adherent and non-adherent fractions; and at higher levels (123-269 copies/μL) from whole PBMC, and whole PBMC inoculated with viruses pre-incubated with convalescent plasma containing SARS-CoV antibodies. However, none of the PBMC preparations were positive for minus-strand viral RNA. The results for the PBMC obtained from the other two SARS-CoV strains Su10 and LC2 were similar.

Apoptosis
DNA fragmentation analysis
DNA fragmentation was observed for Vero cells since day 1. DNA smearing was observed in a few test preparations, but without DNA fragmentation as expected from apoptosis. All tested cell preparations—including lymphocyte cell lines (Jurkat, HSB2, MOLT3, and SupT1) and PBMC

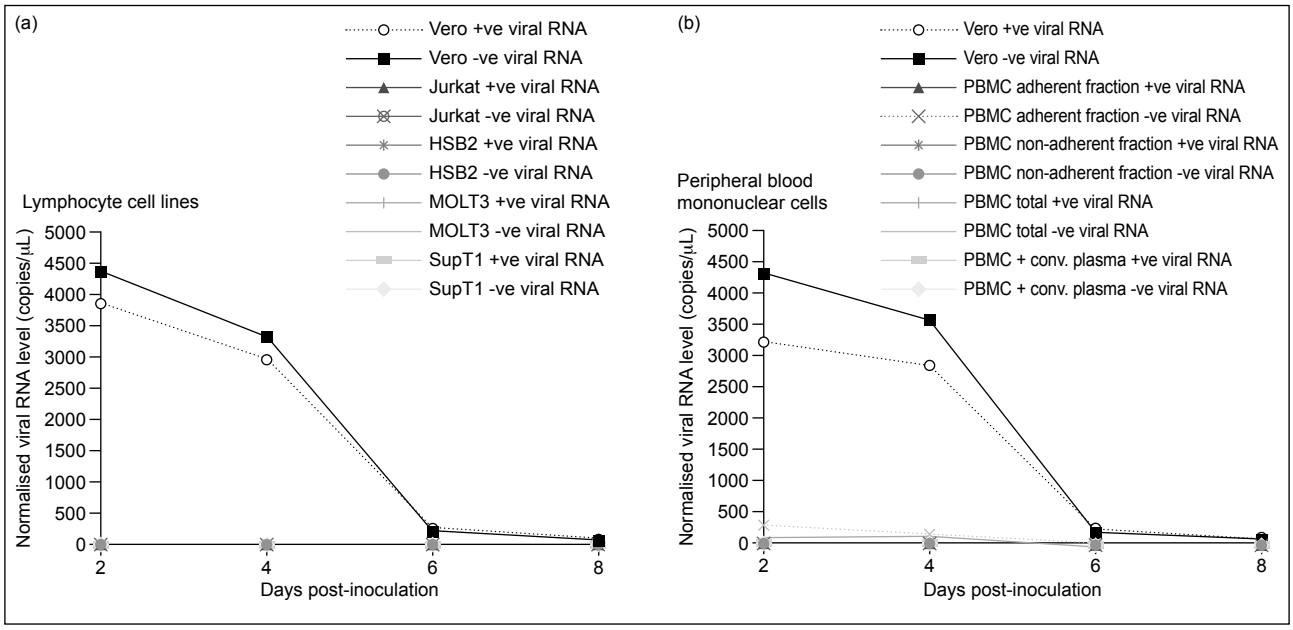

Fig 1. Intracellular SARS coronavirus (SARS-CoV) RNA levels following inoculation with live SARS-CoV
SARS CoV N-gene RNA copy number was obtained by real-time polymerase chain reaction using primers specific respectively for the plus-(genomic) and minus-(replicate intermediate) strand. Normalised viral RNA copy numbers were obtained by dividing the crude viral copy number by the GAPDH RNA copy number obtained from the sample preparation. Data shown are averages of triplicate experiments obtained from virus inoculums of 10 multiplicity of infection of SARS-CoV CUHK-W1, with intracellular viral RNA measured at day-2, -4, -6, and -8 post-inoculation for (a) T lymphocyte cell lines—Jurkat, HSB2, MOLT3, and SupT1; and (b) peripheral blood mononuclear cells (PBMC)—adherent (monocytes) fraction, non-adherent (lymphocytes) fraction, whole PBMC, whole PBMC treated with SARS-CoV pre-incubated with a convalescent plasma containing SARS-CoV antibodies

(adherent/non-adherent fractions, whole PBMC with and without convalescent plasma pre-incubation with viruses) challenged with live SARS-CoV, heat-inactivated virions, and synthetic spike protein fragment—did not show definite evidence of DNA fragmentation.

TUNEL assay

Vero cells started to exhibit intense apoptosis (mean, 26%) since day-2 post-inoculation, which increased dramatically over the next 3 days reaching 72% in day 5. Jurkat, HSB2 and MOLT3 cells exhibited a similar low level of apoptosis throughout the 8 days, and with apoptotic cells ranged 1-6% for Jurkat, 0.7-7% for HSB2, and 0.3-9% for MOLT3; whereas SupT1 showed the lowest level of 0.3-1.6%. The levels of apoptosis were similar for all four preparations of the PBMC (0.6-5% for PBMC adherent fraction, 0.3-5% for PBMC non-adherent fraction, 0.6-4% for whole PBMC, and 1-3% for PBMC with virus pre-incubated with SARS-CoV antibodies). In all lymphocyte cell lines and PBMC fractions tested, no significant difference in the proportion of apoptotic cells between the virus-inoculated and the mock-infected preparation was observed (Fig 2).

Annexin V/PI staining

A small proportion of apoptotic cells (<5%) were observed for all cell lines and the PBMC preparations throughout the 8-day post-inoculation. The proportions of apoptotic cells were similar between the virus-inoculated preparations and their corresponding mock-infected controls.

The proportion of Annexin V-positive but propidium iodide–negative cells following treatment with inactivated SARS-CoV virions, and the synthetic receptor-binding fragment of SARS-CoV spike protein was determined by flow cytometry. A low level of apoptosis (<5%) was observed throughout the 8 days following treatment for all preparations tested, and similar low levels of apoptosis were also noted for the corresponding negative controls. A similar pattern of results ensued when the synthetic receptor-binding SARS-CoV spike protein was used to treat the cells (Fig 3).

Discussion

The most direct pathway to produce lymphopaenia is for the virus to infect lymphocytes, followed by replication leading to cell death via apoptosis or necrosis. The SARS-CoV receptor angiotensin-converting enzyme 2 is expressed in tonsil lymphocytes, and CD209L is expressed in Jurkat cells and lymph nodes. However, whether PBMC are permissive to SARS-CoV infection is unclear. Some have concluded that SARS-CoV replicated poorly in PBMC, others have found evidence of viral replication in PBMC of SARS patients.[5]

To investigate whether human T lymphoid cell lines and PBMC are permissive to SARS-CoV replication, different

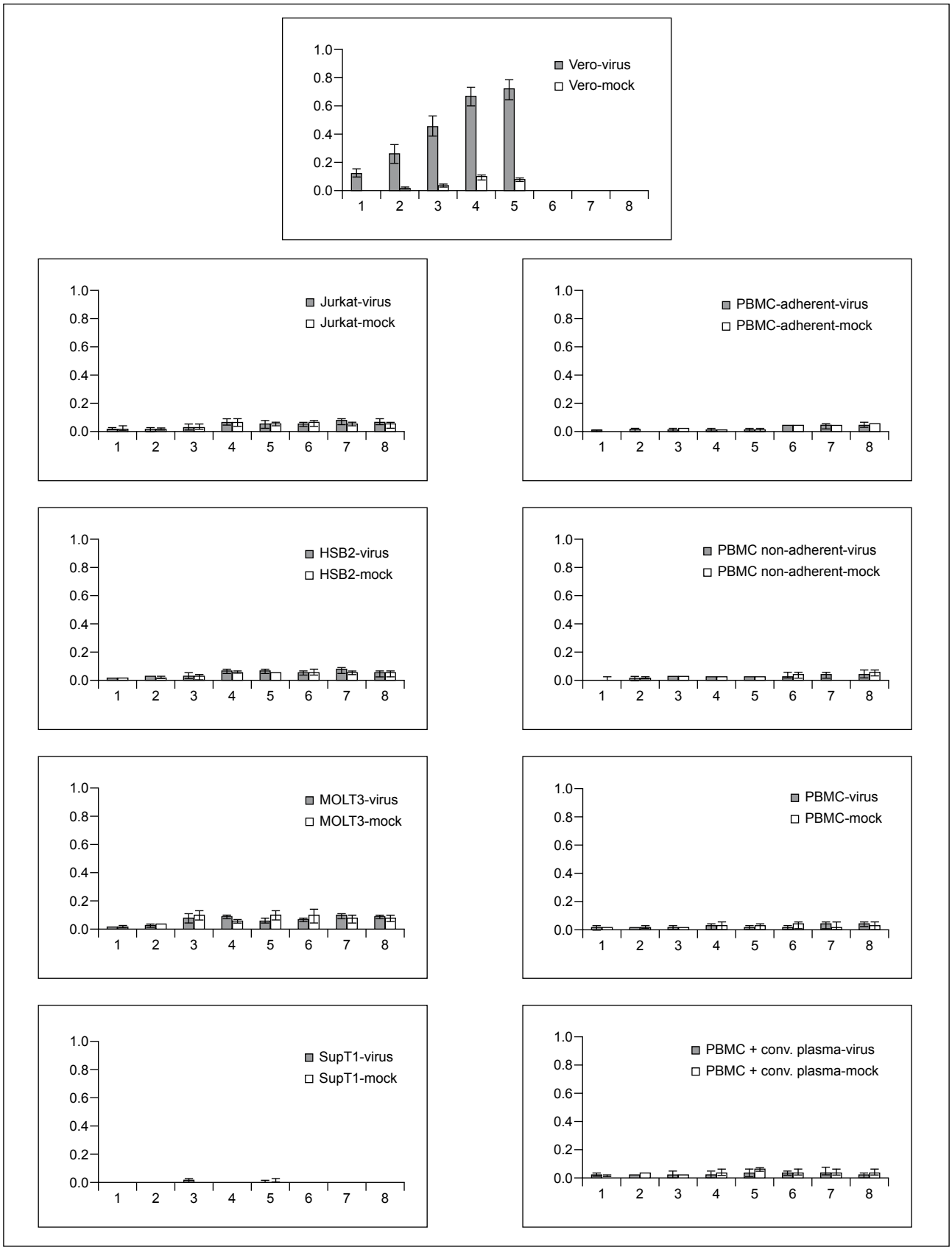

Fig 2. Apoptotic nuclei staining by TUNEL assay after infection with live SARS coronavirus (SARS-CoV)
Y-axis: proportion of cells with apoptotic nuclei detected by TUNEL assay obtained by direct counting. X-axis: harvest time in days post-inoculation of SARS-CoV. Grey bars are results from cells inoculated with live viruses, and white bars are the corresponding mock infection. Results shown are the averages of triplicate experiments with the standard deviations indicated by the error bars. Most of the Vero cells were lysed as a result of viral infection from day 6 onwards, and therefore TUNEL assay was not performed

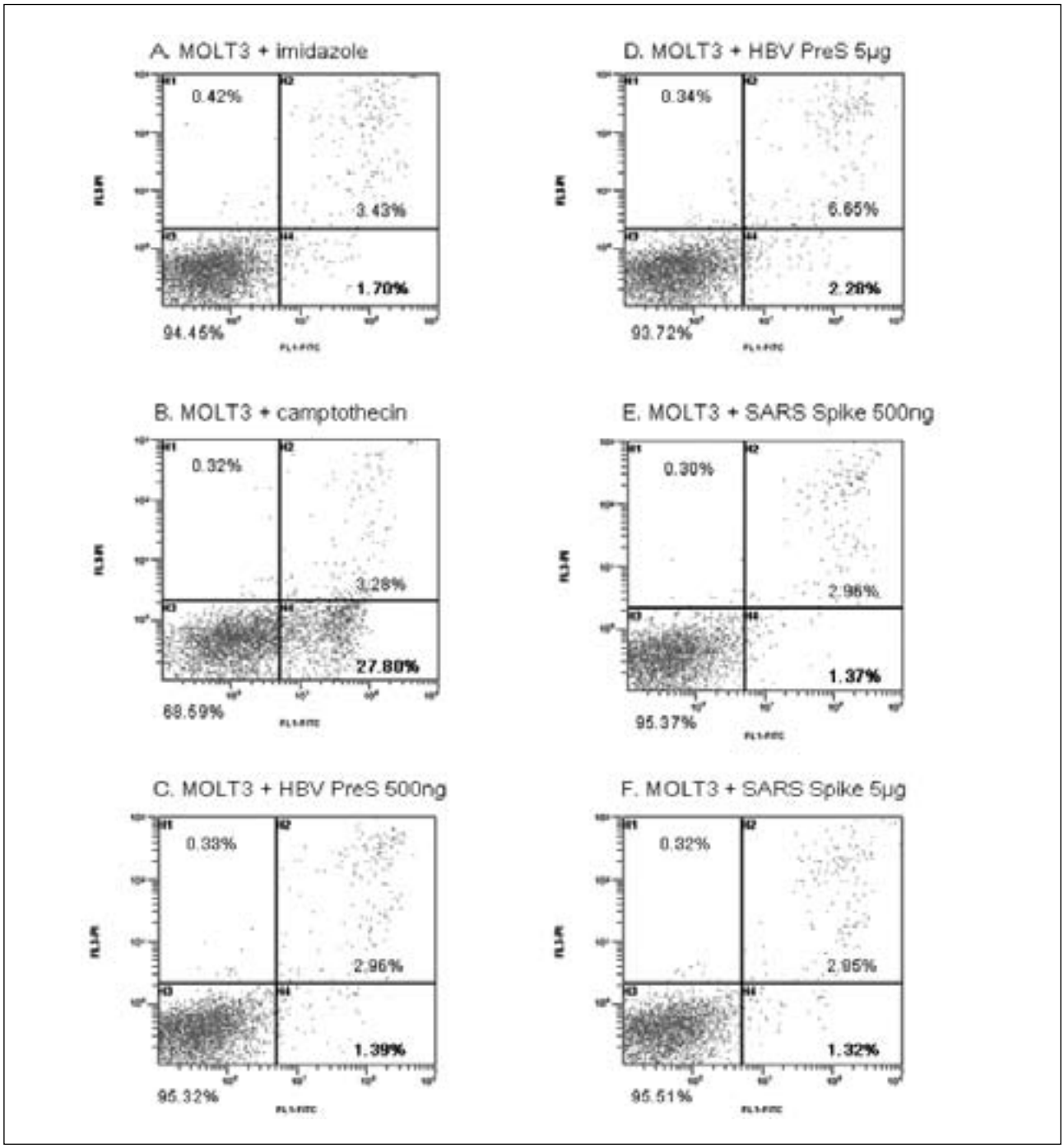

Fig 3. Flow cytometric determination of apoptotic cell population

Duel-labelled quadrants of bi-variant fluorescent dot plots showing proportions of cells stained with annexin V and propidium iodide (PI). Left lower quadrant (H3) represents live cells with low levels of annexin V and PI. Right lower quadrant (H4) represents apoptotic cells with high levels of annexin V. Right upper quadrant (H2) represents secondary necrotic cells with high levels of annexin V and PI. Proportions of cells in each quadrant is shown in percentage with apoptotic cells in bold. Representative data shown were from MOLT3 cells harvested at day-5 post-treatment with: (a) imidazole, a buffer used for the SARS-spike protein, to serve as a negative control; (b) camptothecin, an apoptosis-inducing agent to serve as positive control; (c) pre-S protein of hepatitis B virus synthesised by the same methods as for the SARS-CoV spike protein, to serve as negative control; (d) similar to (c) at a higher dose; (e) synthetic receptor-binding fragment of SARS-CoV spike protein; (f) similar to (e) at a higher dose

strains and doses of SARS-CoV were used. We observed a low, but non-sustained, level of viral genomic RNA; and the minus-strand RNA representing replication was only detected at modest level in Jurkat cells. The intracellular viral RNA quantitation and viral nucleocapsid protein staining results concurred. The low levels of viral RNA and

protein observed might be a result of passive adsorption or update of virions, or low levels of abortive infection; but without active replication of SARS-CoV. We have observed a small proportion of the PBMC showing a low level of signal, which could represent viral protein. This is in line with a recent observation that Fc gamma II receptor can mediate the entry of SARS-CoV into B cells. However, pre-incubating the viruses with antibodies to allow for possible antibody-mediated update, only resulted in a modest increase in signals; still no sustained viral replication could be detected. This implies that the virus may bypass the cell entry step, for example by way of virus-antibody complex binding with Fc receptors, as the intracellular environment of PBMC may not favour productive replication of SARS-CoV.

The absence of productive infection does not exclude the possibility that virions or their components can trigger apoptosis of lymphocytes/monocytes. Interaction between host cells and SARS-CoV in the absence of productive infection has been demonstrated. Chemokine/cytokine induction by SARS-CoV in monocytes/macrophages/dendritic cells has been shown in the absence of productive infection. We therefore carried out further work to determine whether treating lymphocytes/monocytes with infectious SARS-CoV, heat-inactivated virions, and a synthetic receptor-binding fragment of spike protein could result in apoptosis of lymphocytes/monocytes. The occurrence of apoptosis was documented by multiple methods. Using the crude method of DNA fragmentation (as revealed by agarose gel electrophoresis), none of the treated preparations, except the Vero cell control, showed clear evidence of DNA fragmentation. This by itself could still be a false-negative result, due to lack of sensitivity. The results of TUNEL assay also did not show significant increase in apoptosis of treated cells. We then reconfirmed the results using an alternative method to detect phosphatidylserine exposed to extracellular environment in viable cells to indicate apoptosis. The results were quantitated using flow cytometry which is highly sensitive. The levels of apoptosis observed in treated cells were very low, and comparable to those from control preparations. Overall, the same results were observed for the three representative strains CUHK-W1, -Su10 and LC2, under low, moderate, and high doses of virus inoculation.

Conclusions

Our data showed that human lymphocytes/monocytes were not permissive to productive replication of SARS-CoV. Challenge of lymphocytes/monocytes with infectious viruses, inactivated virions, or the receptor-binding fragment of spike protein did not result in apoptosis of corresponding cells. Virus-specific antibodies might enhance the update of SARS-CoV into PBMC. However, the efficiency seemed to be low, and did not result in sustained productive viral replication. These data suggest that the lymphopaenia observed in SARS-CoV infection in humans is a result of indirect mechanisms. Vascular cell adhesion molecule-1 (sVCAM-1), soluble Fas ligand (sFasL), and glucocorticoids as suggested previously may play a role.[3,5] Furthermore, it has been well documented that SARS patients undergo a phase of intense cytokine storm, which can itself induce apoptosis in lymphocytes/monocytes. Further studies on the mechanisms of lymphopaenia in SARS-CoV infection should focus on these possible indirect mechanisms leading to apoptosis or necrosis of lymphocytes.

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