The major histocompatibility complex class I immunopeptidome of extracellular vesicles

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Extracellular vesicles (EVs) are released by most cell types and have been associated with multiple immunomodulatory functions. MHC class I molecules have crucial roles in antigen presentation and in eliciting immune responses and are known to be incorporated into EVs. However, the MHC class I immunopeptidome of EVs has not been established. Here, using a small-scale immunosolation of the antigen serotypes HLA-A*02:01 and HLA-B*27:05 expressed on the Epstein-Barr virus-transformed B cell line Jesthom and MS of the eluted peptides from both cells and EVs, we identified 516 peptides that bind either HLA-A*02:01 or HLA-B*27:05. Of importance, the predicted serotype-binding affinities and peptide-anchor motifs did not significantly differ between the peptide pools isolated from cells or EVs, indicating that during EV biogenesis, no obvious editing of the MHC class I immunopeptidome occurs. These results, for the first time, establish EVs as a source of MHC class I peptides that can be used for the study of the immunopeptidome and in the discovery of potential neoantigens for immunotherapies..

Results

In this study we have performed small-scale MHC class I immunosolation and peptide extraction to determine the repertoire of peptides from EVs of the EBV-transformed B cell line Jesthom, providing the first data that EVs can be used as a source for immunopeptidomic studies of MHC class I epitopes in health and disease.

The EBV-transformed B cell line Jesthom expresses HLA-A*02:01 and HLA-B*27:05. Three small-scale immunosolation experiments were performed from ~250, 600, and 800 million Jesthom cells, yielding ~200, 300, and 400 μg of EVs, respectively. Importantly, cell lysates and EVs from the same culture
were processed concomitantly to prevent temporal sample variations due to dissociation of low affinity peptides. EVs were isolated by standard procedures of filtration and ultracentrifugation. Immunoblot analysis of a sample of the cell and EV lysates indicated enrichment of MHC class I, and the prototypical EV markers CD9 and CD81 in the EV isolates, whereas the non-EV marker GRP78 (BiP) was present at very low levels in the cell lysate and EV isolates. Furthermore, the MHC class I allele (HLA-A*02:01, HLA-B*27:05) is prominent in both cells and EVs. Immunoblot analysis of a sample of the cell and EV lysates was used to confirm the enrichment of the MHC class I molecules, and the low molecular mass peptide pool fraction isolated with Centricon 3 centrifugal filters. The isolated peptide fraction was then processed and analyzed by mass spectrometry.

Identified peptides from the resulting MASCOT files between 8 and 13 amino acids long were analyzed for HLA-A*02:01 and HLA-B*27:05 predicted binding affinity using the NetMHCCons 1.1 server, which combines three algorithms (NetMHC, NetMHCpan, and PickPocket). From the combined data of the three biological replicates 145 and 94 peptides were identified from HLA-B*27:05 cell and EV preparations, respectively, and 172 and 105 peptides from HLA-A*02:01 cell and EV preparations, respectively (Table 1 and supplemental Table S1). In addition 11 HLA-C*01:02 (also expressed by Jesthom cells) peptides were also identified but are not reported here due to low numbers. The mean predicted binding affinity of the HLA-A*02:01-binding peptide pool was 33.3 nM for cells and 26.7 nM for EVs (Fig. 2A), and for the HLA-B*27:05-binding peptide pool the mean predicted affinity was 225.9 nM for cells and 199.5 nM for EVs (Fig. 2D). Although this might suggest that there is a loss of some lower affinity peptides during the biogenesis of EVs, two-tailed Mann-Whitney tests indicated no significant differences between the cell and EV pools for either MHC class I allele (HLA-A*02:01, p = 0.8329, and HLA-B*27:05, p = 0.3199, respectively). Peptide lengths did not alter, with a predominance of 9-mer peptides in both cells and EV for both HLA-A*02:01 and HLA-B*27:05 (Fig. 2B and D). The anchor binding motifs were also analyzed by Seq2Logo for 9-, 10-, and 11-mer peptide pools from each source, with no significant alterations between the typical P2 Leu and C-terminal Val/Leu anchors for HLA-A*02:01 (Fig. 2E) and the P2 Arg and C-terminal Phe/Tyr anchors for HLA-B*27:05 (Fig. 2F). 26 of the 94 HLA-B*27:05 peptides in the EV pool were not detected in the cell-derived pool, and 34 of the 105 HLA-A*02:01 peptides from EVs were not detected in the cell-derived pool (highlighted in red in Table 1), however, the cellular origin of these peptides was mostly from the cytoplasm and nucleus, suggesting they would also appear in the cell-derived pool in a larger sample size. One HLA-A*02:01-binding peptide (LLLD-VPTAAV) was identified from the endosome-located thiol-reductive GILT, but this has previously been reported in cells and therefore unlikely to be EV-specific (14). Taken together the data indicate that the MHC class I immunopeptidome of EVs is a replica of that found on the cell surface.

**Discussion**

Our data has several important implications. It demonstrates that the EV immunopeptidome is essentially identical to that of the cell of origin. As such, important antigenic peptides, such as viral or tumor-associated antigens and tumor-specific antigens were identified in both cell and EV samples (Fig. 1C), which were HC10 reactive (Fig. 1D). Additional bands were detected migrating at the dye front, which were presumed to be β2-microglobulin (Fig. 1C). The remaining 98% of the immunoslated cell and EV samples were acidified in 0.5% TFA to denature the MHC class I molecules, and the low molecular mass peptide pool fraction isolated with Centricon 3 centrifugal filters. The isolated peptide fraction was then processed and analyzed by mass spectrometry.

The immunopeptidome of extracellular vesicles

**Figure 1. Characterization of Jesthom EV.** A, cell lysates and EVs from Jesthom cells were immunoblotted for MHC class I (HC10), GRP78, CD9, and CD81. B, representative nanoparticle tracking analysis of culture supernatant after 0.2-μm filtration, but prior to ultracentrifugation. C, Coomassie Blue-stained SDS-PAGE gel of ~2% of the washed W6/32 immunosolation beads. Control beads (ctrl) received just lysis buffer. The W6/32 was not cross-linked and elutes from the beads with the MHC class I. D, immunoblot of ~2% of the washed W6/32 immunosolation beads, using anti-HLA-B and -C antibody HC10. The second stage anti-IgG also recognizes the IgH and IgL from W6/32 used in the immunosolation step as indicated in C.
# Table 1

Peptide identification and predicted binding affinities

The sequence of the identified peptides for cell and EV derived peptides eluted from HLA-A*02:01 and HLA-B*27:05 are shown in alphabetical order. Peptides in red are those detected in the EV pool only. Predicted binding affinities were generated using NetMHCcons 1.1.

| B27 cells | nM   | B27 EV | nM   | A2 cells | nM   | A2 EV | nM   |
|-----------|------|--------|------|----------|------|-------|------|
| ARFLTTGF  | 36.12| ARFLTTGF| 36.12| AIAPFIAAV| 13.2| AIAPFIAAV| 13.2|
| ARFLEGQV | 123.99| ARFPEPAF| 277.63| AISGIIMGI| 107.14| AIVDKVPSV| 15.45|
| ARFPEPAF  | 277.63| ARFSPDGQYQ| 143.49| AIVDKVPSV| 15.45| AILADGVQKV| 8.2|
| ARFSPDGQYQ| 143.49| ARIALLPLL| 34.77| ALAAALAH| 17.88| ALAGHQDGTIT| 24.2|
| ARIALLPLL  | 34.77| ARILPNVPVL| 122.66| ALADGVQKV| 8.2| ALDQVPDF| 13.42|
| ARIFQFQNF  | 361.9| ARLFIFETF| 113.1| ALDSQPVK| 13.42| ALDTHVDPV| 38.54|
| ARILPNVPVL | 122.66| ARLKEVLEY| 302.73| ALFQRPPPL| 53.61| ALLAYTLG| 5.1|
| ARLIFETF   | 113.4| ARLOQALLV| 89.62| ALIEKLV| 6.06| ALLDRIVS| 3.8|
| ARLKEVLEY  | 302.73| ARNKAITS| 147.43| ALAYTLGV| 5.1| ALMDEVVKA| 7.6|
| ARLPVNSY   | 113.1| ARNPVVS| 243.82| ALMPVLNQV| 4.52| ALMPVLNQV| 4.52|
| ARLOQALLV  | 89.62| ARSPVAPAR| 306.02| ALPPVLTV| 12.71| ALSDSIHTV| 4.26|
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The immunopeptidome of extracellular vesicles

17086 J. Biol. Chem. (2017) 292(41) 17084 –17092
### Table 1—continued

The immunopeptidome of extracellular vesicles

| B27 cells | nM       | B27 EV | nM       | A2 cells | nM       | A2 EV | nM       |
|-----------|----------|--------|----------|----------|----------|-------|----------|
| GRSAF1G1GF| 107.14   | 243.82 | GLDGPPPTV| 51.34    | KLGNVINN| 16.84 |
| GRSEV1YYN | 265.87   | 291.48 | GLDRNAPS | 59.73    | KLLDPEDVAVQ | 24.73 |
| GRTVE5F1L | 212.98   | 471.75 | GLDQRQTV | 10.81    | KLSGSLVAKL| 54.48 |
| GRTF1QM1M | 249.16   | 111.88 | GLDGVLIN | 18.87    | LLDSAPLNV | 20.14 |
| GRTL1V1NY | 133.75   | 170.61 | GLDGNAVE | 8.2      | LLDPVTA4V | 28.78 |
| GRSV1A5AF | 254.61   | 233.74 | GLDQVQTV  | 44.6     | LLGGPVPGV | 72.97 |
| GRSF1K5SY | 147.43   | 37.71  | GLWEIEINPTV| 21.03    | LLNQVSL | 8.34 |
| GRTV1G5SNK | 531.38  | 87.7   | GLWQGQVPTA| 51.06    | LLDDPPVTA | 7.73 |
| GRTV1TTNR | 222.4    | 54.19  | GQIEIVPVE | 4.15     | LLLAPPA | 245.15 |
| GRWS1G5LY | 54.78    | 134.47 | HIIEINAV  | 18.17    | LMDHTIEPV | 3.8  |
| GRYQ1PVLY | 37.71    | 115.57 | HLEEPIYL | 7.6      | LMVDHVTEV | 3.55 |
| GRYA1GQ0GY| 80.87    | 484.69 | HLSTINEL| 138.16   | MLPPPLTA | 263.01 |
| GRYP1GVSNY| 193.22   | 31.38  | ILDKKVKEK| 75.79    | QSDEVFIQL| 23.94 |
| GRYQ1V5WSL | 34.77   | 71.79  | ILDQKINE | 13.42    | QVTDIEK | 21.25 |
| HRAQ1Y1TR | 54.48    | 270.22 | ILDVTVVL | 31.89    | RLGQSPTL | 50.51 |
| HRDS1T1NL | 1091.15  | 110.08 | ILTDIITKV | 37.71    | RLSQAVTV | 16.31 |
| HRFEQ1AF1TY | 47.34  | 522.82 | ILTETINTV| 11.98    | RLPQAEVE | 8.52 |
| HRFY1GK5SSY | 122   | 83.54  | IMLEALERV| 5.38     | RLQPDDPGV | 44.6 |
| HRIK1D1HYSY | 253.23 | 189.08 | IQDNHDGTYTV| 409.85   | RLQEPQAPGV | 42.25 |
| HRT11K1RF | 211.83   | 50.78  | KGTYFVRV | 212.98   | RLQEEINEV | 9.91 |
| HRLL1P1VTSF | 98.79  | 759.4  | KIYEGQVE | 7.81     | SIIGRLE | 18.27 |
| HRNEV1TEL | 388.27   | 337.32 | KLADFQDVAQL| 52.18    | SLADQNDE | 35.34 |
| HRYG1DGGSTF | 108.72 | 719.41 | KLQENIAQL | 26.25    | SLEAVGVLQ | 35.15 |
| IRAA1P1PLF | 243.82   | 26.25  | KLDDQDNEV | 11.05    | SLAQYLIN | 4.65 |
| IRLP15QYFN | 291.48   | 18.47  | KLFGMIIT | 8.07     | SLAQYNK | 20.47 |
| IRNHS1H5QVR | 471.75 | 14.55  | KLGSVPVTVT| 21.96    | SLAVADLF | 17.59 |
| KRAALQ1ALK | 27.71   | 14.48  | KLDDQVQVL| 11.66    | SLDQPTGTV | 91.58 |
| KRFA1DEG1TYVVR | 74.57 | 104.28 | KLIPQUTL | 6.03     | SLHIDIQLSL | 24.47 |
| KRFEH1AKL | 54.48    | 66.56  | KLLDISELDMV| 9.81     | SLINGVGLS | 11.47 |
| KRFEQ1E1AKK | 170.61 | 32.77  | KLDDPEDVAVQ| 24.73    | SLDDPDP VE | 3.31 |
| KRFG1AY1NL | 22.56   | 9.39   | KLQGYPSSL | 6.94     | SLLDRFLATV | 5.8  |
| KRFK1EANNF | 234.76   | 133.03 | KLNPQQFEV | 6.71     | SLLLENK | 21.03 |
| KRFK1V1D5VEGF | 260.18 | 38.33  | KLYAGAIL | 4.67     | SLLEVENESTV | 27.11 |
| KRFK1VAV1NL | 37.71   | 67.28  | KVLQGVVG | 13.13    | SLLGQDDVSV | 9.49 |
| KRHN1V1RKV | 149.84   | 67.65  | LLDRFLATV | 9.54     | SLPLEGPAI | 28.47 |
| KRDI1D1HN | 46.57    | 21.49  | LLDDPVTA | 28.78    | SLPPDLAVGL | 51.89 |
| KRLD1NT1NYY | 134.47 | 28.62  | LLGPPPVGV | 72.77    | SSLQTLYKV | 6.75 |
| KRLD1TVQ5SF | 60.71   | 55.08  | LLIDDKGTVK| 84.9     | SLMLTVEL | 12.51 |
| KRLT1L1VTY | 44.12    | 90.6   | LLNQVSL | 8.34     | SLSPIYPAA | 41.12 |
| KRMNP1SP55TY | 81.75 | 141.18 | LLIPGALTA| 13.57    | SLWQPAEA | 51.61 |
| KRND1V1MHL | 69.88    | 102.05 | LLSSGLPIT | 5.87     | SLVDYNPNL | 3.43 |
| KRPNP1V1KG5Y | 484.69 | 192.18 | LLLDDPVTA | 32.59    | SMADILPGLG | 16.39 |
| KRQA1X1TAF | 98.79    | 236.04 | LLMLDVMI | 37.51    | SMDADVPLV | 5.47 |
| KRTP1S1ETV | 995.27   | 328.32 | LLPEGPAI | 25.55    | SVIEQIVVY | 6.06 |

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### Table 1—continued

| B27 cells | nM  | B27 EV       | nM  | A2 cells | nM  | A2 EV       | nM  |
|-----------|-----|--------------|-----|----------|-----|--------------|-----|
| KRYAVPSAGL| 33.48 | SRLGLPLL     | 31.38 | LLPPAPPHA | 245.15 | TLFDIEVRL | 8.57 |
| KRYKSTTVVY| 71.79 | SRLNEGATY    | 1229.06 | LMDHTIPAV | 3.8 | TLWDPVEV | 6.4 |
| LRFPEVPSKF| 95.12 | SRTSVPQTF    | 437.34 | LMDVHTEV | 3.55 | TLYEAVREV | 11.17 |
| LRFQSSAVMA| 270.22 | SRTVYVTY    | 700.21 | MLFPGSIAL | 4.78 | TVLPPFSSTV | 40.68 |
| LRNPILAKG| 110.08 | SRVKLILEY    | 166.06 | MLPPPLTA | 263.01 | VLDYQRNV | 11.53 |
| LRNSQSFN| 522.82 | SRYQGVNLY    | 181.07 | MLYDIVMV | 2.78 | VLGESINSV | 6.43 |
| NRFPAGGIGGL| 83.54 | TRYQGVNLY    | 384.09 | NLSFIEQV | 10.87 | VLIEITLVL | 10.75 |
| NRLPILVS| 141.95 | YRFLQPQIYLY | 63.39 | NLIDLDDLY | 11.6 | VLPKLQPL | 10.75 |
| QRAVIERF| 189.08 | NLPKLHIV     | 17.21 | VMDSKIVQV | 10.46 |
| QRPFLSFGF| 50.78 | NMAKVDEV    | 57.51 | YLADVTVN | 3.51 |
| QRHSFQISL| 105.42 | QLDDQVEQI | 53.32 | YLDPAQRGV | 24.87 |
| QRIDLAVL| 116.83 | QLQEXPQTL | 11.41 | YLDGQSERV | 7.24 |
| QRILYTVTY| 175.29 | QLVDIEIKV | 21.25 | YLPALVHI | 3.76 |
| QRNIVVEL| 759.4 | QVFPGLLERV | 186.06 | YLLTHPPPPIM | 31.89 |
| QRODIAFAY| 337.32 | RLFDQAGFL | 4.02 | YLPEDFIRV | 3.99 |
| QQTDLTVL| 719.41 | RLLIESVT | 6.82 | YLTNEQGQY | 11.6 |
| QRVSIFDYD| 595.31 | RLDDYVVN | 3.74 | YLYCTFISL | 5.35 |
| QRYNPPS| 89.62 | RRLEDYPYL | 4.02 | YQVQGLYSV | 3.72 |
| RQTGVNL| 187.05 | RLMSNTEAV | 6.94 | YTPVNPNAV | 33.3 |
| RRAKLDADRY| 71.41 | RLPEAIEV | 8.52 |
| RRAQLQQYQQR| 34.59 | RLQEDPPAGV | 44.6 |
| RRDVQKVVG| 207.3 | RLQEDPPVG | 42.25 |
| RRRFPPYVV| 41.8 | RLQEENEV | 9.91 |
| RRRFSPPPPLSY| 26.25 | RLWGEVPNL | 30.54 |
| RRFFVNVPFT| 14.55 | RLYPWPVVEV | 6.5 |
| RRIKEIVKHH| 104.28 | RQLEEEGTTF | 64.08 |
| RRESSQVDRYY| 66.56 | RNFENVAV | 10.07 |
| RRILSDQVVTGY| 133.03 | RVIQSTLEEV | 48.11 |
| RRMPFPPQGH| 44.4 | RVLDPSMVIEV | 47.34 |
| RRMPFPQGHR| 67.65 | SIIGRILLEV | 18.27 |
| RRMPFQGHR| 21.49 | SILEDPPSI | 64.43 |
| RRWLPQGDA| 16.22 | SLAEVAGLVQ | 35.15 |
| RRYYFGTEDRL| 90.6 | SLAQYLIN | 4.65 |
| SRASKVKNF| 917.7 | SLAGPYK | 20.47 |
| SRFPMEAY| 192.18 | SLDPVPQTV | 91.58 |
| SRFQGTLYL| 56.28 | SLHDQLQSL | 24.47 |
| SRFSLENF| 361.9 | SLNIGLSV | 11.47 |
| SRFFPNQALVF| 331.89 | SLDPVTEV | 3.31 |
| SRLAYHNY| 328.32 | SLLDRFLATV | 5.8 |
| SRLATUNEK| 198.52 | SLLLENIK | 21.03 |
| SRLGPLLLL| 31.38 | SLEVNEESTV | 27.11 |
| SRLPSLGAGF| 197.45 | SLLGGDVQSV | 9.49 |
| SRLSFEYTG| 60.06 | SLLPEGPAI | 28.47 |
| SRLVYAYQF| 52.74 | SLLPPDVALG | 51.89 |
### Table 1—continued

| B27 cells | nM  | B27 EV | nM  | A2 cells | nM  | A2 EV | nM  |
|-----------|-----|--------|-----|----------|-----|-------|-----|
| SRNAQTGF  | 191.14 |       |     | SLLQTLKYV | 6.75 |       |     |
| SRNEGVATY | 1229.06 |       |     | SLMLYTVEL | 12.51 |       |     |
| SRNGVITQY | 405.44 |       |     | SLPDFGSIYSV | 6.22 |       |     |
| SRNSNTWVFVK | 723.31 |       |     | SLSPIYPPAA | 41.12 |       |     |
| SRSNTQPQGF | 543 |       |     | SLSQTTPRPRV | 75.79 |       |     |
| SRTSVQPTF | 437.34 |       |     | SLWQGQPAEA | 51.61 |       |     |
| SRVKLILEY | 166.06 |       |     | SLVDYNPNI | 3.43 |       |     |
| SRWKEKVQQR | 93.59 |       |     | SLYGTTITI | 19.28 |       |     |
| SRYQGVNL | 181.07 |       |     | SMADIPLGFGV | 16.39 |       |     |
| TRLQPSAYAK | 979.25 |       |     | SMSADVPLV | 5.47 |       |     |
| TRSQAIFTK | 238.6 |       |     | SMYDKVLML | 10.58 |       |     |
| TRYQGVNL | 384.09 |       |     | SQTFVNHIV | 358.01 |       |     |
| VRFYIESISY | 267.31 |       |     | SVIEEQVYV | 6.66 |       |     |
| YRFFLGNQF | 42.71 |       |     | TLFDYEVR | 8.57 |       |     |
| YRLGNVDAFQ | 61.37 |       |     | TLJEDILGV | 4.36 |       |     |
|          |      |       |     | TLJGLSIV | 15.61 |       |     |
|          |      |       |     | TLWDOFYEVE | 6.4 |       |     |
|          |      |       |     | TLYEAVRE | 11.17 |       |     |
|          |      |       |     | VLFFENTSVH | 21.72 |       |     |
|          |      |       |     | VLIDVGTGYV | 10.07 |       |     |
|          |      |       |     | VLIDYQRNV | 11.53 |       |     |
|          |      |       |     | VLIEGSINSV | 6.43 |       |     |
|          |      |       |     | VLIPKLQL | 10.75 |       |     |
|          |      |       |     | VLLDAPQQL | 8.43 |       |     |
|          |      |       |     | VLLGKVYYV | 6.43 |       |     |
|          |      |       |     | VLMTEDIKL | 54.78 |       |     |
|          |      |       |     | VLODIQVHL | 21.72 |       |     |
|          |      |       |     | VLDWRDTS | 3.43 |       |     |
|          |      |       |     | VMDSKIQVQ | 10.46 |       |     |
|          |      |       |     | YLADVTNAL | 3.51 |       |     |
|          |      |       |     | YLGQVTITI | 52.74 |       |     |
|          |      |       |     | YLJEPDVEL | 5.99 |       |     |
|          |      |       |     | YLLQHILI | 4.4 |       |     |
|          |      |       |     | YLEQTEQQA | 25.55 |       |     |
|          |      |       |     | YLLPAIVHI | 3.76 |       |     |
|          |      |       |     | YLLQEPPTV | 13.57 |       |     |
|          |      |       |     | YLPEDFRV | 3.99 |       |     |
|          |      |       |     | YLSKIIPI | 4.29 |       |     |
|          |      |       |     | YLTHDSPSV | 5.29 |       |     |
|          |      |       |     | YLTNEGQY | 11.6 |       |     |
|          |      |       |     | YLYPDITRL | 4.06 |       |     |
|          |      |       |     | YQVQLYSV | 3.72 |       |     |
are likely to be released in EVs, potentially subverting antigen-specific CD8+ cytotoxic T cells at a distance from the infected cell or main tumor, thus potentially reducing effective CTL responses. The same observation would, however, also imply that EVs can be used as an effective source to isolate and detect tumor-specific antigens and tumor-associated antigens from...
readily available biological samples such as blood. EVs are known to be raised in pathological conditions (15, 16), suggesting a relatively non-invasive technique for screening. As such, the EV-derived peptidome can now be studied as a source for neoantigens for personalized immunotherapeutic approaches, as recently demonstrated in principle for melanoma solid tissue biopsies (17). Such identified peptides could then be utilized in dendritic cell exosome-based therapies (18), for which phase I and II trials have already been conducted. Furthermore, this MS technique could be used to monitor the efficacy of target-peptide loading onto dendritic cell exosomes.

Our study does have some limitations. Our current small-scale study yielded a few hundred peptides, but larger samples and improved detection could yield thousands of identified peptides. The small, but consistent alteration in predicted binding affinities in the EV peptide pool would be worth studying in greater detail with such larger sample sizes. The residency time of an MHC class I complex during its incorporation into and secretion via an EV would be expected to promote the loss of low-affinity peptides. Larger sample sizes would help resolve this issue. Furthermore, variations in the biogenesis of MVBs in different cell types could also have a significant impact upon the EV immunopeptidome. An extensive study of multiple cell types is now required. Of technical interest, the antibody-based immunosorption of MHC molecules for peptide isolation utilized here is just one of several possible techniques (19). We have also performed preliminary studies on EV samples using mild acid elution (MAE), which in theory would not disrupt the cell or EV samples. The MAE technique removes the detergent lysis, immunosorption, and extensive washing steps that could lead to loss of low-affinity peptides. However, MAE is known to produce increased peptide signals of non-MHC origin (20), but intriguingly we were able to detect some peptides with known binding motifs for the MHC class II molecules expressed on Jesthom cells (data not shown), thus further enhancing the capacity of EVs to produce useful immunopeptidomic information. Taken together our study opens a new avenue for the characterization of the immunopeptidome from highly biologically relevant vesicles.

**Experimental procedures**

**Cell and EV isolation**

The EBV-transformed B cell line (obtained from the European Collection of Authenticated cell Lines no. 88052004) was grown in RPMI 1640 (Invitrogen, UK) supplemented with 5% FBS (Invitrogen, UK). Once the required number of cells was obtained, the medium was replaced with serum-free medium to prevent contamination from FBS-derived exosomes (EX-Cell 610-HSF serum-free, Sigma-Aldrich, UK) for 48 h. Cells were then isolated by centrifugation (300 × g, 10 min). The cells were washed once in PBS, then immediately resuspended in 5 ml of lysis buffer (1% Nonidet P-40, 150 mM NaCl, 10 mM Tris, pH 7.6, with 1 mM PMSE). After 10 min on ice the lysates were centrifuged at 20,000 × g for 5 min and the supernatant stored on ice. 10–20 μl was removed for immunoblotting.

The EV containing supernatant was processed immediately by 0.2-μm filtration and ultracentrifugation at 100,000 × g for 2 h. The pellets were resuspended in 500 μl of PBS and 10–20 μl was removed for BCA protein estimation or EV characterization by immunoblotting. The remaining bulk EV suspension was immediately lysed in 5 ml of lysis buffer, as above.

**EV characterization**

Nanoparticle tracking analysis was performed on a 0.5-ml cell culture sample after 0.2 μm filtration. Videos were taken using a Nanosight LM-10 unit (Malvern, UK), with a 4-ms camera shutter and analysis detection threshold of 2 using NTA 2.3 software. For immunoblotting, 5 μg of cell or EV lysates were run on 4–20% gels (Invitrogen) and transferred to nitrocellulose. Samples were incubated with the following antibodies overnight, HC10 (anti-HLA-B and -C), HCA2 (anti-HLA-A, a gift from Jacques Neefjes, Leiden), anti-GRP78 (dilution 1:5000, code STJ97526, St. Johns Laboratories, UK), or anti-CD9 or -CD81 (Thermo Fisher Scientific UK, codes: anti-CD9 clone Ts9, dilution 1:5000, code 15328354; and CD81, dilution 1:5000, code 15304032). Immunoblots were then incubated with 1:10,000 diluted IR Dye800cw anti-mouse IgG (LI-COR, UK, code 925-32210) and visualized using a LI-COR Odyssey scanner.

**Immunosorption of MHC class I peptides**

0.5 ml of Protein G-agarose (code 20399, binding capacity 11–15 mg/ml of IgG, Thermo Scientific UK) were pre-loaded with 30 ml of W6/32 containing tissue culture supernatant for 20 min at room temperature, then washed twice in lysis buffer. The beads were then added to the cell and EV lysates and mixed for 1 h at 4 °C. Control W6/32-loaded beads received lysis buffer alone. The beads were then washed with 60 volumes (3 × 10 ml) of lysis buffer without Nonidet P-40. 10 μl of beads was then removed for reducing SDS-PAGE and Coomassie Blue staining (Gelcode Blue, Thermo Scientific, UK). The remaining beads were resuspended in 1 ml of 0.5% TFA for 10 min at room temperature. The supernatant was then spun at 12,000 × g through pre-washed (in 0.1% TFA) Centricron 3 filtration units (MerckMillipore, UK). The peptide containing flow-through was then stored at −20 °C until analysis by mass spectrometry.

**Mass spectrometry**

Peptides were concentrated using a C18 column (NEST, Thermo Scientific UK), eluted in 70% acetonitrile, 0.5% TFA and dried down by SpeedVac. Peptides were then analyzed on an AB Sciex TripleTOF 5600+ system mass spectrometer (Sciex, Framingham, MA) coupled to an Eksigent nanoLC AS-2/2Dplus system. The samples were loaded in loading buffer (2% acetonitrile and 0.05% trifluoroacetic acid) and bound to an Aclaim pepmap 100 μm × 2-cm trap (Thermo Fisher Scientific), and washed for 10 min to waste after which the trap was turned in-line with the analytical column (Aclaim pepmap RSLC 75 μm × 15 cm). The analytical solvent system consisted of buffer A (2% acetonitrile and 0.1% formic acid in water) and buffer B (2% water with 0.1% formic acid in acetonitrile) at a flow rate of 300 nl/min with the following gradient: linear 1–20% of buffer B over 90 min, linear 20–40% of buffer B for 30 min, linear 40–99% of buffer B for 10 min, isocratic 99% of buffer B for 5 min, linear 99–1% of buffer B for 2.5 min, and
The immunopeptidome of extracellular vesicles

isocratic 1% solvent buffer B for 12.5 min. The mass spectrometer was operated in the DDA top 20 positive ion mode, with 120 and 80 ms acquisition time for the MS1 (m/z 400 –1250) and MS2 (m/z 95 –1800) scans, respectively, and 15-s dynamic exclusion. Rolling collision energy was used for fragmentation. Peak lists were generated within PeakView by using the “create mgf file” script. The MASCOT search engine with the following search parameters was used to identify peptides: no enzyme specificity, maximum of 4 misscleavages, oxidation as variable modification, peptide tolerance was set to 20 ppm, and the MS-MS tolerance to 0.1 Da. Data were searched against Swiss Prot database downloaded November 2016, restricted to proteins from humans only.

Peptides identified in the Mascot files were assessed for their potential allele-binding specificity and affinity using the NetMHCcons 1.1 algorithm. Alleles HLA-A*02:01, -B*27:05, and -C*01:02 were selected for screening, using standard settings of 0.5% Rank and IC₅₀ 50 nm for strong binders, and 2% Rank and IC₅₀ 500 nm for weak binders. Peptides identified as HLA-A*02:01, -B*27:05, and -C*01:02 binders were tabulated, and Mann-Whitney two-tailed tests were performed using Prism 7 software. Anchor motifs were analyzed using Seq2Logo algorithm (22) (www.cbs.dtu.dk/biotools/Seq2Logo/), using the Shannon format and standard settings.

Author contributions—S. J. P. and C. B. designed and obtained the funding for the study. S. J. P. and F. G. M. C. isolated the peptides. S. A. S. and S. L. S. performed the mass spectrometry and data analysis. S. J. P. performed the MHC algorithm data. A. N. A. and S. J. P. wrote the first draft of the manuscript, with all authors contributing to the final version.

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