Supporting Information

Proteolysis by Granzyme B Enhances Presentation of Autoantigenic Peptidylarginine Deiminase 4 Epitopes in Rheumatoid Arthritis

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SI Methods

MS/MS peptide mapping for HDX

Intact or GrB-cleaved PAD4 was incubated in reaction buffer (7.5mM Tris, 150mM NaCl, 0.4mM EDTA, 4% glycerol, 4mM β-mercaptoethanol) prepared with H_2O for peptide mapping, mixed with HDX quench buffer (1°C, 3M urea, 1% v/v trifluoroacetic acid), digested with a pepsin-coupled column at 1°C (Applied Biosystems POROS 20 AL, bead size 20 μm, column size 1 mm x 20 mm), and analyzed by reversed-phase HPLC coupled with ESI MS and MS/MS, using an automated workflow [32]. An MS/MS peptide coverage map of PAD4 was generated in order to provide a peptide set for subsequent HDX MS experiments (Figure S-1). For MS/MS peptide mapping, digest was separated on Agilent 1100 HPLC equipped with a C8 trap and a C18 column (2 mm i.d. x 50 mm, Thermo Fisher Scientific) using a 45-min gradient at flow rate 50 μL/min (buffer A 0.3% v/v formic acid, buffer B 80% v/v acetonitrile 0.3% v/v formic acid, linear increase from 5% B to 100% B over 45 min). Collision-induced dissociation (CID) MS/MS spectra were acquired for the five most abundant ions from each MS scan on a linear ion trap LTQ mass spectrometer equipped with ESI source (Thermo Fisher Scientific), as described [32]. Peptides were identified by searching MS/MS spectra against PAD4 sequence using Mascot software (Matrix Science), without protease specificity or protein modification. Only peptides with a Mascot score greater than 20 and further verified by high-resolution MS were considered for HDX peptide set.

Cell-free MHC class II antigen processing system mass spectrometry analysis

For LCMS/MS experiments to identify DR1 binding epitopes, lyophilized peptides were re-constituted in 1μL of 50% acetonitrile/0.1% TFA and diluted with 9μL 2% acetonitrile/0.1% formic acid before LCMS/MS analysis using LTQ Orbitrap Velos MS (Thermo Scientific) interfaced with nanoAcquity UPLC (Waters). Peptides were loaded on a 75 μm x 2.5 cm C18 (YMC*GEL ODS-A

12nm S-10 µm) trap at 600 nl/min 0.1% TFA and fractionated at 300 nL/min on a 75 µm x 150 mm ProntoSIL-120-5-C18 H reverse-phase column (5µm, pore size 120Å, from BISCHOFF Chromatography) using a 2-90% acetonitrile gradient in 0.1% formic acid over 85min. Eluting peptides were sprayed into the mass spectrometer through 1 µm emitter tip (New Objective) at 2.2 kV. Survey scans (full ms) were acquired at 350-1800 m/z, up to 8 peptide masses (precursor ions) were individually isolated within 1.9 Da and fragmented using HCD 35 activation collision energy with a 20 sec dynamic exclusion and one repeat count. Precursor and the fragment ions were analyzed at resolution 60,000 and 15,000, respectively using the 371 m/z siloxane ion for a lock mass. Isotopically resolved masses in precursor (MS) and fragmentation (MS/MS) spectra were extracted with and without deconvolution using Xtract or MS2 Processor nodes in Proteome Discoverer (v1.4, Thermo Scientific). All extracted data were searched using Mascot software (Matrix Science) through Proteome Discoverer against the sample's species proteins in RefSeq 2012 database. Proteome Discoverer uses only the peptide identifications with the highest Mascot score for the same peptide spectrum from the three different ion extraction nodes. The following criteria were set for all database searches: sample's species; no enzyme; precursor and fragment mass tolerance 10 ppm and 0.2 Da, respectively; cysteine carbamidomethylation fixed modification; methionine oxidation variable modification. Peptide identifications from Mascot searches were filtered in Proteome Discoverer to identify peptides with a 1% False Discovery Rate confidence threshold, based on a concatenated reverse database search.

SI Figures

Figure S-1

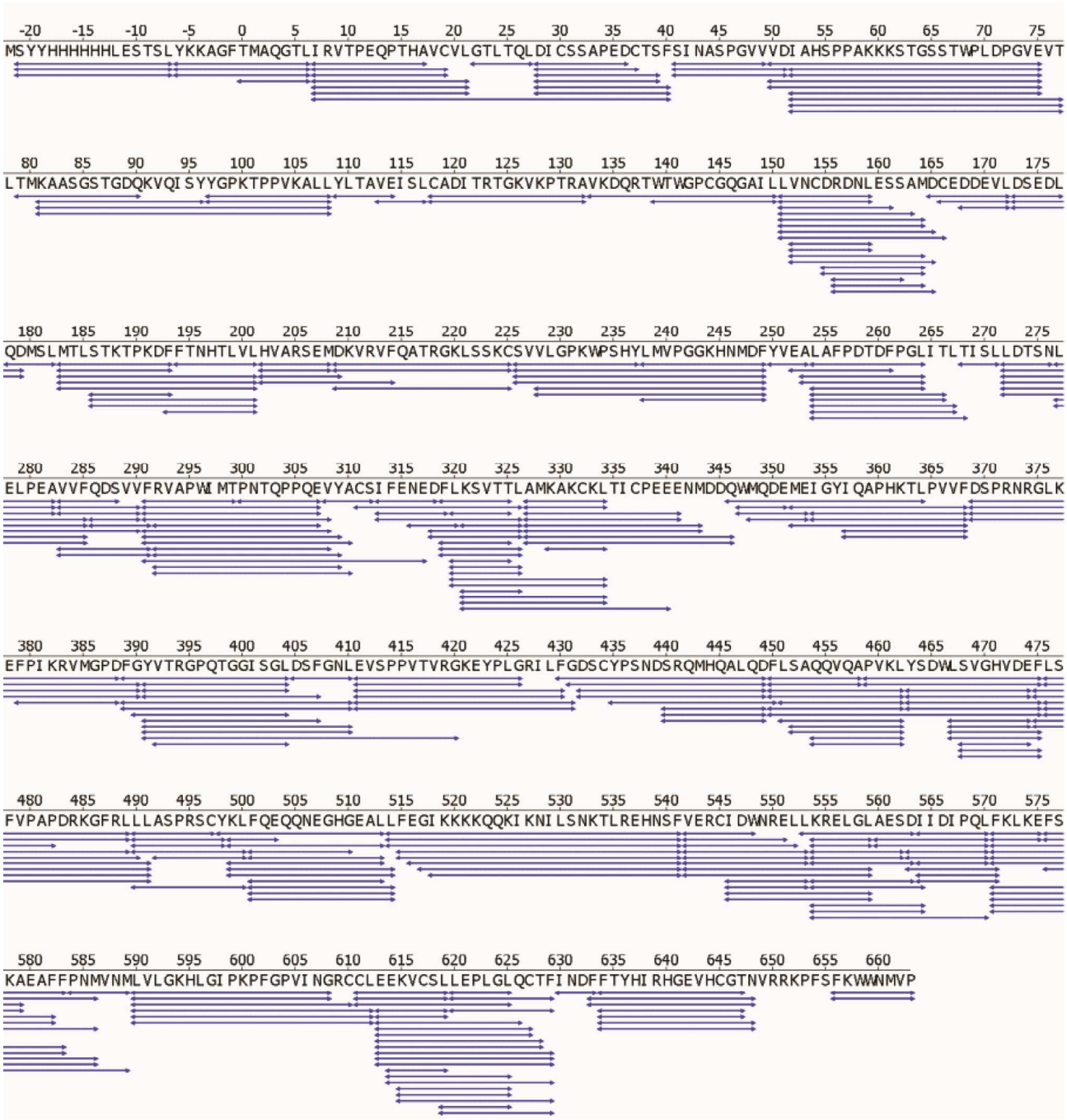


Figure S-1. Sequence coverage map of intact PAD4 peptides. Intact PAD4 was digested with a pepsin column and analyzed by HPLC MS/MS. Sequences were confirmed by the MASCOT program and only those with a score >20 and further verified by high-resolution MS were included for subsequent HDX analysis.

Figure S-2

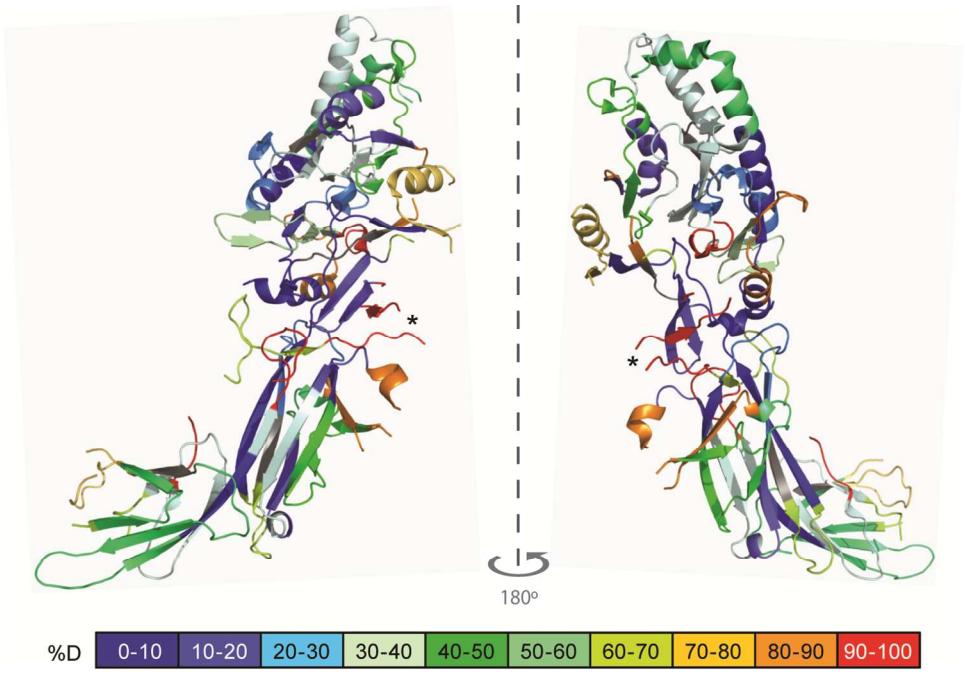
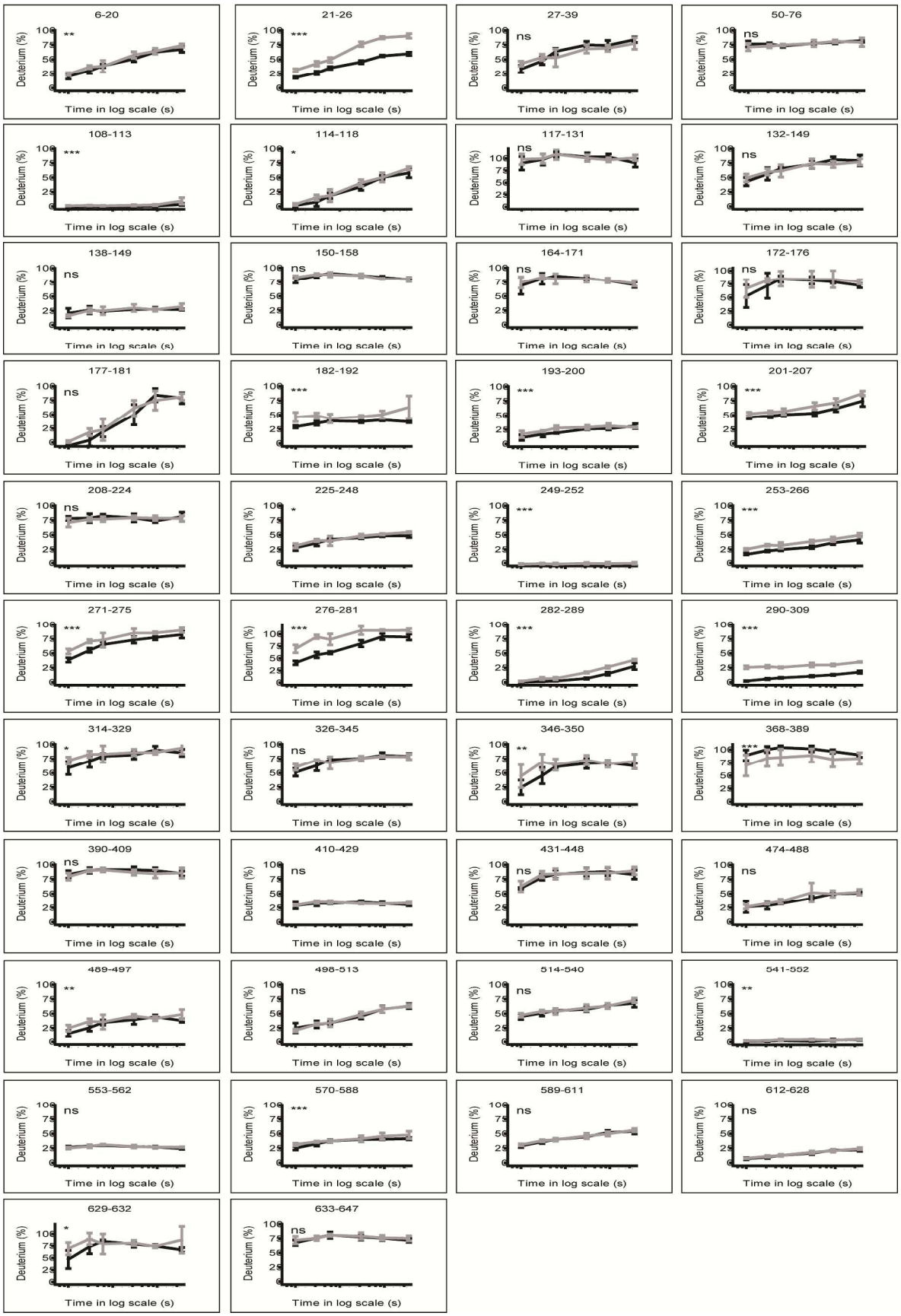


Figure S-2. The HDX profile of intact PAD4 reflects the globular dimeric nature predicted by the crystal structure. The % incorporation of deuterium (%D) is shown for all of the peptides identified from intact PAD4 and is overlaid onto the calcium-free crystal structure of the PAD4 monomer (PDB ID 1WD8) [34]. The GrB cleavage site at D388 is indicated (*) and was too unstructured to obtain crystallographic data.


6-20
**
21-26
***
27-39
ns
50-76
ns
108-113
***
114-118
*
117-131
ns
132-149
ns
138-149
ns
150-158
ns
164-171
ns
172-176
ns
177-181
ns
182-192
***
193-200
***
201-207
***
208-224
ns
225-248
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249-252
***
253-266
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271-275
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276-281
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282-289
***
290-309
***
314-329
*
326-345
ns
346-350
**
368-389
***
390-409
ns
410-429
ns
431-448
ns
474-488
ns
489-497
**
498-513
ns
514-540
ns
541-552
**
553-562
ns
570-588
***
589-611
ns
612-628
ns
629-632
*
633-647
ns
Deuterium (%)
100
75
50
25
0
Time in log scale (s)

Figure S-3. HDX profiles of individual peptides identified from both intact and GrB-cleaved PAD4. HDX data of PAD4 with (—) and without GB cleavage (**—**) is plotted as % incorporation of deuterium as a function of incubation time in log scale (s). Data were analyzed by two-way ANOVA analysis: *** represents $p<0.001$; ** represents $p<0.01$; * represents $p\leq0.05$, and ns represents not significantly different. The error bars were plotted as the standard deviations from four independent HDX experiments.

SI Tables

Table S-1. Relative quantification of the DR1 alpha chain nested peptide set from intact or GrB cleaved PAD4

Peptide identified by cell-free system	Peptide peak area	
	Intact PAD4	GrB cleaved PAD4
NVPPEVTVLTNSPVELREPN	8.427×10^7	5.936×10^7
NVPPEVTVLTNSPVELR	0.780×10^7	1.054×10^7
VPPEVTVLTNSPVELREPN	1.207×10^7	0.553×10^7
PPEVTVLTNSPVELR	0.101×10^7	0.114×10^7
NVPPEVTVLTNSPVELREPNVL	0.675×10^7	1.273×10^7
PPEVTVLTNSPVELREPN	0.249×10^7	0.173×10^7
NVPPEVTVLTNSPVELREPNV	0.312×10^7	0.313×10^7
VPPEVTVLTNSPVELREPNVL	0.077×10^7	0.111×10^7
Total peptide peak area	11.83×10^7	9.527×10^7

Since empty MHC molecules tend to denature and aggregate despite efforts to generate a peptide-free receptive conformation, a portion of sDR1 was bound to peptides derived from the DR1 alpha chain, NVPPEVTVLTNSPVELREPN. The areas calculated for PAD4-derived epitopes were normalized to the total peptide peak area for this internal control peptide.

Table S-2. Relative quantification of the MAQ(1-17) nested set from intact or GrB cleaved PAD4

MAQ(1-17) variants identified by Mass Spectrometry	Total peptide peak area (ion counts x sec)	
	Intact PAD4	GrB cleaved PAD4
YKKAGFTMAQGTLIRVTPEQPTH	0.583×10^7	8.566×10^7
QGTLIRVTPEQPTHAVC	0.446×10^7	0.614×10^7
MAQGTLIRVTPEQPTHA	0.153×10^7	0.272×10^7
KKAGFTMAQGTLIRVTPEQPTH	0.263×10^7	4.014×10^7
GTLIRVTPEQPTHA	0.191×10^7	0.170×10^7
LYKKAGFTMAQGTLIRVTPEQPTH		1.835×10^7
LYKKAGFTMAQGTLIRVTPEQPTHA		1.850×10^7
YKKAGFTMAQGTLIRVTPEQPTHA		16.430×10^7
KKAGFTMAQGTLIRVTPEQPTHA		4.985×10^7
AQGTLIRVTPEQPTHA		0.374×10^7
KKAGFTMAQGTLIRVTPEQPTHA		1.470×10^7
STSLYKKAGFTMAQGTLIRVTPEQPTH		3.157×10^7
LYKKAGFTMAQGTLIRVTPEQPT		1.696×10^7
SLYKKAGFTMAQGTLIRVTPEQPTH		2.832×10^7
YKKAGFTMAQGTLIRVTPEQPTH		1.721×10^7
YKKAGFTMAQGTLIRVTPEQPT		1.280×10^7
KKAGFTMAQGTLIRVTPEQPT		0.459×10^7
YKKAGFTMAQGTLIRVTPEQPTHA		2.499×10^7
LYKKAGFTMAQGTLIRVTPEQPTH		1.311×10^7
TSLYKKAGFTMAQGTLIRVTPEQPTHA		1.404×10^7
Total Peptide peak area	1.636×10^7	56.94×10^7

Table S-3. Relative quantification of ASP(44-61) nested set from intact or GrB cleaved PAD4

ASP(44-61) variants identified by Mass Spectrometry	Peptide peak area (ion counts x sec)	
	Intact PAD4	GrB cleaved PAD4
ASPGVVVDIAHSPPAKKK	4.566×10^7	7.006×10^7
SPGVVVDIAHSPPAKKK	1.105×10^7	2.160×10^7
ASPGVVVDIAHSPPAKKKSTG	0.608×10^7	0.888×10^7
PGVVVDIAHSPPAKKK	1.660×10^7	2.505×10^7
INASPGVVVDIAHSPPAKKK	1.084×10^7	1.884×10^7
SINASPGVVVDIAHSPPAKKK	0.390×10^7	0.758×10^7
ASPGVVVDIAHSPPAKKKST	0.200×10^7	0.354×10^7
PGVVVDIAHSPPAKKKSTG	0.277×10^7	0.546×10^7
ASPGVVVDIAHSPPAKKKSTGSST	0.199×10^7	
DIAHSPPAKKK	0.343×10^7	
ASPGVVVDIAHSPPAKK		0.160×10^7
VDIAHSPPAKKK		0.266×10^7
ASPGVVVDIAHSPPAKKKS		0.546×10^7
Total Peptide peak area	10.43×10^7	17.07×10^7

Table S-4. Relative quantification of LTM(78-93) nested set from intact or GrB cleaved PAD4

LTM (78-93) variants identified by Mass Spectrometry	Total peptide peak area (ion counts x sec)	
	Intact PAD4	GrB cleaved PAD4
VTLTMKAASGSTGDQKVQ	0.241×10^7	0.317×10^7
LTMKAASGSTGDQKVQ	0.841×10^7	1.000×10^7
$VTLTM_{(OX)}KAASGSTGDQKVQ$		0.152×10^7
Total Peptide peak area	1.082×10^7	1.469×10^7

(ox): Oxidation of Methionine

Table S-5. Relative quantification of ISY(94-105) nested set from intact or GrB cleaved PAD4

ISY(94-105) variants identified by Mass Spectrometry	Total peptide peak area (ion counts x sec)	
	Intact PAD4	GrB cleaved PAD4
ISYYGPKTPPVK	11.790 x 10^7	10.570 x 10^7
SYYGPKTPPVK	4.763 x 10^7	3.958 x 10^7
YYGPKTPPVK	0.626 x 10^7	
ISYYGPKTPPVKA		1.315 x 10^7
Total Peptide peak area	17.18 x 10^7	15.84 x 10^7

Table S-6. Relative quantification of IGY(354-368) nested set from intact or GrB cleaved PAD4

IGY(354-368) variants identified by Mass Spectrometry	Total peptide peak area (ion counts x sec)	
	Intact PAD4	GrB cleaved PAD4
APHKTLPVVF	0.214 x 10^7	0.367 x 10^7
IGYIQAPHKTLPVVFDSPRNRG		0.821 x 10^7
IGYIQAPHKTLPVVF		6.324 x 10^7
GYIQAPHKTLPVVF		0.762 x 10^7
Total Peptide peak area	0.214 x 10^7	8.273 x 10^7

Table S-7. Relative quantification of RGP(394-405) nested set from intact or GrB cleaved PAD4

RGP(394-405) variants identified by Mass Spectrometry	Total peptide peak area (ion counts x sec)	
	Intact PAD4	GrB cleaved PAD4
RGPQTGGISGLDSFGNLE	1.016 x 10^7	2.475 x 10^7
RGPQTGGISGLDSFGNL	0.223 x 10^7	
RGPQTGGISGLD		0.429 x 10^7
FGYVTRGPQTGGISGLD		0.879 x 10^7
Total Peptide peak area	1.239 x 10^7	3.783 x 10^7

Table S-8. Relative quantification of QAL(445-461) nested set from intact or GrB cleaved PAD4

QAL(445-461) variants identified by Mass Spectrometry	Total peptide peak area (ion counts x sec)	
	Intact PAD4	GrB cleaved PAD4
QALQDFLSAQQVQAPVK	1.490×10^7	33.03×10^7
HQALQDFLSAQQVQAPVK	0.514×10^7	8.911×10^7
DFLSAQQVQAPVK	1.232×10^7	4.665×10^7
ALQDFLSAQQVQAPVK	0.191×10^7	7.963×10^7
QALQDFLSAQQVQ	0.163×10^7	0.507×10^7
M(ox)HQALQDFLSAQQVQAPVK		2.133×10^7
MHQALQDFLSAQQVQAPVK		0.963×10^7
QDFLSAQQVQAPVK		1.836×10^7
DFLSAQQVQAPVKLYSD		3.386×10^7
FLSAQQVQAPVKLYSD		1.345×10^7
Total Peptide peak area	3.591×10^7	64.74×10^7

(ox): Oxidation of Methionine

Table S-9. Relative quantification of VGH(468-488) nested set from intact or GrB cleaved PAD4

VGH(468-488) variants identified by Mass Spectrometry	Total peptide peak area (ion counts x sec)	
	Intact PAD4	GrB cleaved PAD4
VPAPDRKGFRLL	2.285×10^7	4.054×10^7
VGHVDEFLSFVPAPDRKG	0.285×10^7	0.272×10^7
SFVPAPDRKGFRLL	1.161×10^7	0.808×10^7
SFVPAPDRKGFR	1.441×10^7	1.312×10^7
HVDEFLSFVPAPDRKG	0.145×10^7	0.271×10^7
PAPDRKGFRLL	1.254×10^7	2.214×10^7
PAPDRKGFR	0.771×10^7	0.702×10^7
FVPAPDRKGFR	1.113×10^7	1.031×10^7
PDRKGFR	0.450×10^7	0.315×10^7
SFVPAPDRKGFRL	0.189×10^7	
FVPAPDRKG	0.016×10^7	
VPAPDRKGFRLLLASPRSCYK		0.590×10^7
VGHVDEFLSFVPAPD		0.171×10^7
FVPAPDRKGFRLL		2.140×10^7
Total Peptide peak area	9.110×10^7	13.88×10^7