

**EXPLORING THE IMMUNOMODULATION
PROFILES OF THE THP-1 HUMAN
MACROPHAGE-DERIVED CELL LINE
MEDIATED BY *Shigella flexneri***

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by

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TABLE OF CONTENTS

ACKNOWLEDGEMENT	ii
TABLE OF CONTENTS	iv
LIST OF TABLES	xii
LIST OF FIGURES	xiv
LIST OF SYMBOLS	xxv
LIST OF ABBREVIATIONS	xxvi
LIST OF APPENDICES	xxviii
ABSTRAK	xxix
ABSTRACT	xxx
CHAPTER 1 INTRODUCTION	1
1.1 Background	1
1.2 Problem statement	4
1.3 Research questions	6
1.4 Objectives	6
1.4.1 General objective.....	6
1.4.2 Specific objectives.....	6
1.5 Significance of the study	7
1.6 The layout of the thesis	8
CHAPTER 2 LITERATURE REVIEW	10
2.1 A general overview of <i>Shigella</i> and shigellosis	10
2.1.1 <i>Shigella</i>	10
2.1.2 Shigellosis incidences worldwide	13
2.1.3 Shigellosis incidences in Malaysia.....	15
2.1.4 Transmission of <i>Shigella</i> and clinical manifestation of shigellosis	17

2.1.5	The emergence of multi-drug resistant <i>Shigella</i>	19
2.1.6	Pathogenesis of shigellosis.....	20
2.2	Immune responses against shigellosis.....	23
2.2.1	Innate immunity	24
2.2.2	Adaptive immunity.....	30
2.3	Macrophages and MHC-peptide complex.....	33
2.3.1	MHC of a macrophages.....	35
2.3.2	Presentation of a peptide by macrophages	37
2.4	Shigellosis prevention through vaccination	40
2.5	Outer membrane proteins are the candidate of choice for vaccine development	42
2.6	OMPs as a candidate for epitope-based <i>Shigella</i> vaccine	47
2.7	HLA class II immunopeptidomes analyses to determine the candidate of OMPs-based <i>Shigella</i> vaccine development	48
2.8	Conclusions of literature review	50
CHAPTER 3 METHODOLOGY.....		52
3.1	Preparation of media, buffer, and reagents	54
3.1.1	Preparation of media and reagent for THP-1 cell culture	55
3.1.2	Preparation of media and reagent for bacteriological experiments	56
3.1.3	Preparation of agarose gel.....	57
3.1.4	Preparation of buffers and reagents for immunoprecipitation	58
3.1.5	Preparation of buffers for gene cloning, protein expression, and purification	60
3.1.6	Preparation of buffers for SDS-PAGE and Western blot.....	64
3.1.7	Preparation of buffers for ELISA.....	68
3.2	THP-1 cell culture growth condition.....	69
3.2.1	Cell thawing	69
3.2.2	Changing media and subculturing.....	69

3.2.3	Cell counting	70
3.2.4	Cell differentiation	72
3.3	Gentamicin-inhibitory activity on THP-1-derived macrophages.....	73
3.4	Bacterial strains growth conditions	74
3.4.1	Preparation of bacterial suspensions	75
3.5	Gentamicin-protection assay	75
3.5.1	Infection assay	76
3.5.2	The extracellular killing and intracellular survival of <i>S. flexneri</i> 2a	77
3.5.3	Statistical analysis	78
3.6	Gene expression related to the invasion of <i>S. flexneri</i> 2a into THP-1-derived macrophages.....	78
3.6.1	Infection assay	78
3.6.2	RNA extraction	79
3.6.3	RNA concentration and purity measurement	81
3.6.4	RNA integrity validation by RNA electrophoresis	81
3.6.5	cDNA synthesis.....	82
3.6.6	Primer design for RT-qPCR.....	83
3.6.7	Determination of target genes cDNA expression.....	84
3.6.8	Statistical analysis	86
3.7	Nitric oxide (NO) production assay	86
3.7.1	Pre-treatment	86
3.7.2	Reaction steps.....	87
3.7.3	Statistical analysis	87
3.8	Macrophage-killing assay	88
3.9	Immunoprecipitation of HLA class II-peptide complexes.....	89
3.9.1	Infection assay and lysis of infected THP-1-derived macrophages.....	91

3.9.2	Immunoprecipitation of the target antigen	92
3.9.3	Purification of peptides	93
3.9.4	Desalting.....	93
3.9.5	Protein identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS)	94
3.10	Bioinformatics analysis	96
3.10.1	Protein localization prediction	96
3.10.2	Protein structure analysis	97
3.10.3	Conservation prediction	99
3.10.4	Antigenic B-cell epitope prediction	99
3.10.5	Antigenic T-cell epitope prediction.....	100
3.10.6	Peptide sequence selection from the full-length protein	100
3.11	Cloning of the selected proteins in pET28a	101
3.11.1	Primer design.....	102
3.11.2	Amplification of the FepA and TonB genes	105
3.11.3	Digestion of the DNA (insert) and recipient plasmid (pET28a vector).....	105
3.11.4	DNA extraction from agarose gel	107
3.11.5	Ligation of the digested insert and CIP-treated pET28a vector ...	108
3.11.6	Preparation of TOP10 competent cells.....	109
3.11.7	Bacterial transformation TOP10 competent cells with ligated pET28a-protein vector.....	110
3.11.8	Colony PCR for confirmation of transformed TOP10 competent cells	110
3.11.9	Preparation of overnight culture of transformed TOP10 competent cells and plasmid extraction	111
3.12	Transformation of BL21 (DE3) competent cells, expression, and purification	112
3.12.1	BL21 (DE3) competent cells transformation using the heat-shock method.....	113

3.12.2	Colony PCR of transformed BL21 (DE3) cells	113
3.12.3	Seeding and induction for protein expression	114
3.12.4	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)	115
3.12.5	Cell lysis by sonication	116
3.12.6	Purification of protein	116
3.12.7	SDS-PAGE and Western blotting	117
3.12.8	Protein concentration determination	118
3.13	Immunogenicity test of the synthetic peptide	119
3.13.1	ELISA-peptide assay protocol	119
3.13.2	Dot blot.....	121
3.14	Outer membrane protein and surface-associated proteins preparations.....	123
3.14.1	Preparation OMPs from <i>S. flexneri</i>	123
3.14.2	Surface-associated proteins (SAPs) preparation	124
3.15	Immunization of rats	125
3.15.1	Husbandry	125
3.15.2	Immunization of rat.....	126
3.15.3	Blood collection from the tail vein of rats.....	129
3.15.4	Cardiac puncture blood collection.....	131
3.15.5	Spleen collection	132
3.16	Antibody response of OMPs- and SAPs-immunized rats	132
3.16.1	ELISA.....	132
3.16.2	SDS-PAGE and Western blotting	134
3.17	Gene expression profile of the mediators in the spleen of immunized rats .	135
3.17.1	RNA extraction	135
3.17.2	RNA concentration and purity measurement.....	137
3.17.3	RNA integrity validation by RNA electrophoresis	137
3.17.4	cDNA synthesis.....	138

3.17.5	Primer design for RT-qPCR.....	139
3.17.6	Determination of target genes cDNA expression.....	140
3.17.7	Statistical analysis	142
3.18	Immunogenicity assay of recombinant FepA and TonB proteins against OMPs- and SAPs-immunized rat serum.....	142
3.18.1	ELISA-peptide assay protocol	142
3.18.2	SDS-PAGE and Western blotting	144
CHAPTER 4	RESULT	146
4.1	THP-1 cell culture growth.....	146
4.2	Gentamicin-inhibitory activity on THP-1-derived macrophages.....	148
4.3	Bacterial strains growth.....	150
4.4	Gentamicin-protection assay	152
4.5	Gene expression profile of <i>Shigella</i> -infected THP-1-derived macrophages	155
4.5.1	RNA concentration and purity measurement.....	155
4.5.2	RNA integrity checking	157
4.5.3	Gene expression related to the invasion of <i>S. flexneri</i> 2a into THP-1-derived macrophages.....	159
4.5.4	NO production assay and iNOS gene expression.....	162
4.5.5	HLA class II gene expression.....	164
4.6	Macrophage-killing Assay	166
4.7	Immunoprecipitation	168
4.8	Identification of HLA class II-restricted <i>S. flexneri</i> peptides by LC-MS/MS	168
4.9	Bioinformatic analyses	174
4.9.1	Protein localization prediction	175
4.9.2	Protein structure analysis	178
4.9.3	Conservation prediction	185
4.9.4	Antigenic B-cell epitopes and T-cell epitopes prediction	188

4.9.5	Selection of OMPs fragment from the full-length protein	196
4.9.6	The summary of the bioinformatics analysis	202
4.10	Cloning of the selected proteins in pET28a	204
4.10.1	Primer design.....	204
4.10.2	Amplification of the synthesized primers (XhoI and NcoI primer).....	205
4.10.3	Digestion of the DNA and pET28a	207
4.10.4	Colony PCR for confirmation of transformed TOP10 competent cells.....	209
4.11	Protein expression, purification, and confirmation of the proteins	211
4.11.1	Colony PCR for confirmation of transformed BL21 (DE3) competent cells.....	211
4.11.2	Protein expression and Western blot analysis	213
4.11.3	Purification of recombinant protein	217
4.11.4	Protein concentration determination	219
4.12	Immunogenicity assay of the synthetic peptides.....	222
4.12.1	Recombinant FepA.....	222
4.12.2	Recombinant TonB	226
4.13	Animal immunization.....	230
4.13.1	Antibody immune response against the OMPs and SAPs.....	232
4.13.2	Cytokine gene expression by the immunized rats.....	238
	4.13.2(a) RNA concentration and purity measurement	238
	4.13.2(b) RNA integrity checking.....	239
	4.13.2(c) Determination of gene expression profile by qPCR	241
4.13.3	Immunogenicity assay of recombinant FepA and TonB proteins against OMPs- and SAPs-immunized rat serum IgG.....	245
	4.13.3(a) Immunogenicity assay of recombinant FepA	245
	4.13.3(b) Immunogenicity assay of recombinant TonB.....	249

CHAPTER 5	DISCUSSION	253
5.1	The growth of the THP-1 cell line	255
5.2	Gentamicin inhibitory activity on THP-1-derived macrophages	257
5.3	Bacterial strains growth.....	258
5.4	Gentamicin-protection assay	259
5.5	Gene expression and inflammatory response of infected THP-1-derived macrophages.....	261
5.6	Macrophage-killing assay	267
5.7	Immunoprecipitation and LCMS/MS.....	268
5.8	Immunoinformatic analysis.....	273
5.8.1	Outer membrane proteins selection.....	274
5.8.2	Conservation analysis.....	276
5.8.3	B-cell and T-cell antigenic epitopes prediction.....	277
5.8.4	Selection of OMPs fragment from the full-length protein	278
5.9	Cloning of the selected proteins in pET28a	279
5.10	Protein expression and purification.....	283
5.11	Immunogenicity assay of the synthetic peptides.....	286
5.12	Animal immunization.....	288
5.13	Gene expression assay.....	293
5.14	Immunogenicity assay of recombinant FepA and TonB against immunized-rat serum	294
5.15	Summary of discussion	295
CHAPTER 6	CONCLUSIONS AND RECOMMENDATIONS.....	297
	REFERENCES.....	301
	APPENDICES	

LIST OF TABLES

		Page
Table 2.1	Mediators produced in response to <i>Shigella</i> infection.	27
Table 2.2	The list of OMPs-based vaccine studies against shigellosis.	45
Table 3.1	Preparation of priming premix using the Tetro cDNA Synthesis kit.	82
Table 3.2	Oligonucleotides for qPCR (Annealing temperature 60 °C).	84
Table 3.3	Preparation of oligo reactions using the SensiFAST™ SYBR® Hi- ROX kit.	85
Table 3.4	Preparation of priming premix using the Tetro cDNA Synthesis kit.	139
Table 3.5	Oligonucleotides for RT-qPCR (Annealing temperature 60 °C).	140
Table 3.6	Preparation of oligo reactions using the SensiFAST™ SYBR® Hi- ROX kit.	141
Table 4.1	RNA concentration and purity.	156
Table 4.2	The summary of the single-letter code for each amino acid represented from the LC-MS/MS data.	174
Table 4.3	The subcellular localization prediction of HLA class II-associated peptides derived from the <i>Shigella</i> -infected macrophages ^a	177
Table 4.4	The position of HLA class II-associate peptides in the full-length protein identified using the BLASTp server.	179
Table 4.5	Prediction of antigenicity, the position of the secretory signal peptide, and transmembrane helicase of HLA class II-associated peptides.	182
Table 4.6	The conservation of the selected OMPs with other species of <i>Shigella</i> , <i>Salmonella</i> , <i>Klebsiella</i> , and human homologous.	186

Table 4.7	The potential epitopes of 12 selected outer membrane proteins from <i>S. flexneri</i> 2a that can elicit both B-cell and T-cell immune responses.	189
Table 4.8	Prediction of B-cell epitopes of outer membrane receptor FepA. ...	197
Table 4.9	Prediction of B-cell epitopes of TonB-dependent receptor.....	199
Table 4.10	The selected peptide sequence from the full-length sequence and the corresponding nucleotide sequence.....	201
Table 4.11	Forward (FW) and reverse (RV) primers amplify FepA and TonB sequences. Each primer incorporates a specific restriction site (underlined) at the 5' and 3' of the genes.	204
Table 4.12	Recombinant protein concentration	221
Table 4.13	RNA concentration and purity.	238

LIST OF FIGURES

		Page
Figure 2.1	(a) The morphology of <i>Shigella</i> colonies on SS agar plate. The colonies showed smooth, colorless, without black-centered. (b) <i>Shigella</i> was stained with Gram’s stain and observed under the light microscope with a 40× objective lens. The shape of the bacteria is rod-shaped and stained with pink color, indicating the Gram-negative bacteria.	11
Figure 2.2	The global distribution of <i>Shigella</i> species. Shigellosis caused by <i>S. boydii</i> is not displayed because it is uncommon. Data source: (Bennish & Ahmed, 2020).	14
Figure 2.3	The map shows the monsoons that hit Malaysia every year. Southwest monsoon (----->) arises from May to September, and Northeast monsoon (—>) occurs from October to March. The figure was created with BioRender.com.	16
Figure 2.4	<i>Shigella</i> invasion during shigellosis. The process begins when <i>Shigella</i> invades the epithelial barrier through M cells, followed by the uptake by the resident macrophages. However, <i>Shigella</i> produce a variety of virulent factors to escape the phagosome and induce macrophage apoptosis. The apoptotic macrophages produce cytokines, which leads to the recruitment of PMN cells. This causes the destabilization of the epithelial layer, subsequently allowing the invasion of more <i>Shigella</i> through a basolateral route and dissemination within the mucosa. The figure was created with BioRender.com.....	22
Figure 2.5	The HLA genes in the short arm of chromosome 6. The HLA class I genes compose HLA-A, HLA-C, and HLA-B encoding molecules that present antigens to CD8+ T -cells. The HLA class II genes comprise HLA-DP, HLA-DQ, and HLA-DR encoding molecules that present antigens to CD4+ T-cells. The HLA class	

	III genes encode complement and cytokine molecules. The figure was created with BioRender.com.....	36
Figure 2.6	The central role of macrophages activating helper T-cells in the cell-mediated immune response. Fragment of peptides presented by the macrophages through HLA class II to the helper T-cell leads to the induction of memory cell, B-cell, cytotoxic T-cell, and phagocytic cells. The figure was created with BioRender.com.	39
Figure 2.7	The membrane structure of Gram-negative bacteria and the position of OMPs on the membrane. The illustration was created with BioRender.com	43
Figure 3.1	The flow of the experiments in collecting data in the present study. The figure was created with BioRender.com.....	53
Figure 3.2	Schematic illustration of a hemocytometer. Ten microliters of cell suspension were introduced underneath the coverslip. The viable cells in the 16 corner squares in areas A, B, C, and D were counted.	71
Figure 3.3	The experiments performed to identify antigenic peptides presented by the <i>S. flexneri</i> 2a-infected macrophages. The procedure started with laboratory experiments that involved infection assay, immunoprecipitation, and LC-MS/MS. The bioinformatics analyses were applied to select the sequence of peptides for vaccine candidates of <i>S. flexneri</i> 2a. Finally, the recombinant proteins were synthesized for the immunogenicity assay. The figure was created with BioRender.com.	90
Figure 3.4	Primer design for the fragment from FepA using Primer3Plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).....	103
Figure 3.5	Primer design for the fragment from TonB using Primer3Plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).....	104

Figure 3.6	The flow of restriction enzyme digestion and ligation of the gene of interest and the bacterial plasmid. The figure was created with BioRender.com.....	106
Figure 3.7	The arrangement of different concentrations of recombinant proteins spotted on cellular nitrate. SAPs; surface-associated proteins.....	122
Figure 3.8	Immunization and bleeding schedule of the Sprague Dawley rat. Blood from the tail of the rats was collected just before the first immunization and two weeks after the first and second immunization. A cardiac puncture was performed two weeks after the third immunization. The figure was created with BioRender.com.....	128
Figure 3.9	The cross-section schematic tail of a rat. The blood was collected from the tail vein of the rats. The figure was created with BioRender.com.....	130
Figure 4.1	The differentiation of monocytic THP-1 cells into macrophages. (a) The seeded monocytic THP-1 cells in 24-well plates (1×10^6 cells/well) were cultured in RPMI-1640 with 10% FBS for 24 hours. The cells display a round shape and a nonadherent pattern. In the other well (b), the stimulated THP-1 cells by adding 100 ng/mL PMA for 72 hours. The cells display an elongated shape and an adherent pattern. The cells were snaped using a phase-contrast microscope at the magnification of $\times 200$	147
Figure 4.2	Schematic diagram showing IC_{50} value of gentamicin inhibitory activity towards THP-1-derived macrophages. Ten concentrations of gentamicin (50, 100, 150, 200, 250, 300, 350, 400, 450, and 500 $\mu\text{g/mL}$) were tested for their inhibitory effects on THP-1-derived macrophages by incubation for 24 hours at standard growth conditions. Dose-response data points represent the mean value of triplicates.....	149
Figure 4.3	The morphology of the colonies for <i>S. flexneri</i> 2a (a) mild and (b) virulent strains on SS agar. The bacteria were inoculated on the SS	

agar and incubated at 37°C overnight. The colorless colonies show the bacteria was from the *Shigella* species. 151

Figure 4.4 Extracellular killing and intracellular survival of *S. flexneri* 2a mild strain against 99.62 µg/mL gentamicin in THP-1-derived macrophages. Infected cells were centrifuged at 700 rpm for 10 minutes, followed by incubation for 1 hour at 37°C to permit efficient uptake. Unbound bacteria were removed by washing, and extracellular bacteria were killed by 6, 12, and 24 hours of incubation with gentamicin. The number of extracellular and intracellular bacteria was determined immediately after gentamicin treatment (0 hours) and after a 6, 12, and 24 hours incubation. (a) CFU/mL of extracellular *S. flexneri* 2a mild strain from the supernatant, (b) CFU/mL of intracellular *S. flexneri* 2a mild strain from the lysate. The presented data are the mean and standard deviation from biological and technical triplicates. Statistical analysis was done by Student's *t*-test. A *p* value of less than 0.05 was considered statistically significant. * *p*<0.05; ** *p*<0.01. 153

Figure 4.5 Extracellular killing and intracellular survival of *S. flexneri* 2a virulent strain against 99.62 µg/mL gentamicin in THP-1-derived macrophages. Infected cells were centrifuged at 700 rpm for 10 minutes, followed by incubation for 1 hour at 37°C to permit efficient uptake. Unbound bacteria were removed by washing, and extracellular bacteria were killed by 6, 12, and 24 hours of incubation with gentamicin. The number of extracellular and intracellular bacteria was determined immediately after gentamicin treatment (0 hours) and after a 6, 12, and 24 hours incubation. (a) CFU/mL of extracellular *S. flexneri* 2a virulent strain from the supernatant, (b) CFU/mL of intracellular *S. flexneri* 2a virulent strain from the lysate. The presented data are the mean and standard deviation from biological and technical triplicates. Statistical analysis was done by Student's *t*-test. A *p* value of less than 0.05 was considered statistically significant. * *p*<0.05; ** *p*<0.01. 154

- Figure 4.6 Gel electrophoresis of RNA from *Shigella*-infected THP-1-derived macrophages. The RNAs from (a) N0-N24; Uninfected cells incubated 0-24 hours, P; LPS-infected cells incubated 0-24 hours, and (b) M: mild strain-infected cells incubated 0-24 hours, V: virulent strain-infected cells incubated 0-24 hours were run on 1% agarose gel at 120 V for 30 minutes. The intense bands were 28S ribosomal RNA, and the less intense bands at the bottom were 18S ribosomal RNA. 158
- Figure 4.7 The bar chart shows the gene expression of (a) TNF α , (b) IL-1 β , (c) IL-6, (d) IL-12, and (e) IL-10 by THP-1-derived macrophages infected individually with *S. flexneri* 2a mild strain and virulent strain. The THP-1-derived macrophages (1×10^6 cells/well of 24-well culture plate) were infected with *S. flexneri* 2a at an MOI of 10. qPCR was performed for 40 cycles, and the Ct values from each sample were compared with GAPDH. The presented data are the mean and standard deviation from biological and technical triplicates. Statistical analysis was done by two-way ANOVA. A *p* value of less than 0.05 was considered statistically significant. * *p*<0.05; ** *p*<0.01; *** *p*<0.001. Error bars represent standard deviation..... 161
- Figure 4.8 The bar chart shows the (a) NO production and (b) iNOS gene expression by THP-1-derived macrophages infected with *S. flexneri* 2a mild strain and virulent strain. THP-1-derived macrophages (1×10^6 cells/well of 24-well culture plate) were infected with *S. flexneri* 2a at MOI of 10. The supernatant of THP-1-derived macrophages infected with *S. flexneri* 2a for 0, 6, 12, and 24 hours was used to determine NO concentration by using NO assay kit E-BC-K036 (Elabscience, USA), while the pellet was used to determine the iNOS gene expression by using the qPCR method. The qPCR was performed for 40 cycles, and the Ct values from each sample were compared with GAPDH. The presented data are the mean and standard deviation from biological and technical triplicates. Statistical analysis was done by two-way

	ANOVA. A <i>p</i> value of less than 0.05 was considered statistically significant. * <i>p</i> <0.05; ** <i>p</i> <0.01; *** <i>p</i> <0.001. Error bars represent standard deviation.	163
Figure 4.9	The bar chart shows the HLA class II gene expression by THP-1-derived macrophages infected with <i>S. flexneri</i> 2a mild and virulent strain. The THP-1-derived macrophages (1×10^6 cells/well of 24-well culture plate) were infected with <i>S. flexneri</i> 2a at an MOI of 10. The RT-qPCR was performed for 40 cycles, and the Ct values from each sample were compared with GAPDH. The presented data are the mean and standard deviation from biological and technical triplicates. Statistical analysis was done by two-way ANOVA. A <i>p</i> value of less than 0.05 was considered statistically significant. * <i>p</i> <0.05; ** <i>p</i> <0.01; *** <i>p</i> <0.001. Error bars represent standard deviation.	165
Figure 4.10	Comparative intracellular macrophage killing assay on <i>S. flexneri</i> 2a-infected THP-1-derived macrophages. The assay was performed on the lysate after the time-dependent phagocytosis.	167
Figure 4.11	The distribution of eluted HLA class II-associated peptides retrieved from the LC-MS/MS. The bar chart shows the number of peptides relative to control.	169
Figure 4.12	Venn diagram represents the number of overlapped HLA class II peptides eluted from <i>Shigella</i> -infected macrophages infected with the mild and virulent strains incubated for 5 hours and 12 hours. ...	171
Figure 4.13	A bar chart of peptide lengths showed as a percentage of the whole pool for the mild and virulent strains of <i>S. flexneri</i> 2a.	173
Figure 4.14	Protein localization prediction using PSORTb version 3.0.2 of <i>S. flexneri</i> 2a infection of THP-1-derived macrophages. (a) 5 hours incubation of mild strain-infected macrophages (b) 12 hours incubation of mild strain-infected macrophages (c) 5 hours incubation of virulent strain-infected macrophages (d) 12 hours incubation of virulent strain-infected macrophages.	176

Figure 4.15	The summary of the bioinformatic analysis in selecting peptides to develop the epitope-based <i>Shigella</i> vaccine.....	203
Figure 4.16	PCR amplification for the selected genes using the primers carrying NcoI and XhoI at the 5' and 3' end. The FepA and TonB were identified as a single band at 555 bp 537 bp, respectively. The PCR product was run at a volume of 10 μ L on 1.5% agarose gel at 90 V for 50 minutes.	206
Figure 4.17	Agarose gel electrophoresis of double digestion for pET28a, (a) FepA and (b) TonB. The PCR product was run at a volume of 10 μ L on 1.5% agarose gel at 90 V for 50 minutes.....	208
Figure 4.18	Agarose gel electrophoresis of the gene of interest cloned in pET28a. Lane 1: DNA ladder, lane 2: PCR product of FepA gene as a control, lane 3: recombinant of pET28a-FepA plasmid, lane 4: PCR product of TonB gene as a control, lane 5: recombinant of the pET28a-TonB plasmid. Products were electrophoresed at the volume of 10 μ L on 1.5% agarose gel and ran at 90 V for 50 minutes.	210
Figure 4.19	Agarose gel electrophoresis of the colony PCR for transforming BL21 (DE3) competent cells with recombinant pET28a-protein vector Lane 1: DNA ladder, Lane 2: recombinant of pET28a-FepA plasmid, Lane 3: recombinant of the pET28a-TonB plasmid. Products were electrophoresed at the volume of 10 μ L on 1.5% agarose gel and ran at 90 V for 50 minutes.....	212
Figure 4.20	12% SDS-PAGE analysis (a) recombinant FepA and (b) recombinant TonB proteins for 0 hours and 4 hours in BL21 (DE3). M: Protein marker (kDa). T0: Total protein before induced with IPTG (0 hours). T4: Total protein after induced with IPTG and incubated at 37°C for 4 hours. CE: Crude extract of the protein (supernatant).....	214
Figure 4.21	Western blot analysis of expressed recombinant (a) FepA and (b) TonB proteins for 0 hours and 4 hours in BL21 (DE3). The membrane was stained with Ponceau S stain (the protein bands	

	stained with red color) and then incubated with 1:5000 mouse Anti-Histag as primary antibody and antibody anti-mouse HRP as a secondary antibody (the protein band stained with dark grey). M: protein markers (kDa). T0: Total protein before induced with IPTG (0 hours). T4: Total protein after induced with IPTG and incubated at 37°C for 4 hours.	216
Figure 4.22	SDS-PAGE of purified proteins of (a) FepA and (b) TonB with Ni-NTA resin. M: protein markers (kDa). T0: Total protein before induced with IPTG (0 hours). T4: Total protein after induced with IPTG and incubated at 37°C for 4 hours. W1 - W5: Different fractions of wash. E1.1 - E2.7: Different fractions of elution.....	218
Figure 4.23	Standard curve of BSA concentration (mg/mL). Two-fold dilutions of 1 mg/mL were prepared and subjected to the Bradford method. The absorbance was read at 595 nm wavelength and was prepared in triplicates. Based on the absorbance reading, a standard curve was generated using a formula of $y=mx+c$	220
Figure 4.24	Dot blot analysis was employed to determine the reaction of various concentrations of recombinant FepA with the antibody in the serum. The recombinant FepA was blotted on nitrocellulose membrane at 1 μ L at different concentrations (0.025 mg, 0.1 mg, 0.25 mg, 0.5 mg, and 1.47 mg). Peptides were spotted on a nitrocellulose membrane and exposed to serum and secondary antibodies. SAPs acted as the positive control.....	223
Figure 4.25	ELISA analysis of recombinant FepA at OD 450 nm using the clinical and commercialized sera at 1:100 concentration. The results were expressed as optical density (OD). Each experiment was performed in triplicates. The normal human and rabbit serums were responsible for the negative control.	225
Figure 4.26	Dot blot analysis to determine the reactivity of different concentrations of recombinant TonB with the antibody in the serum. The recombinant TonB was blotted on nitrocellulose membrane at 1 μ L at different concentrations (0.025 mg, 0.1	

	mg, 0.25 mg, 0.5 mg, and 1.47 mg). Peptides were spotted on a nitrocellulose membrane and exposed to serum and secondary antibodies. SAPs acted as the positive control.....	227
Figure 4.27	ELISA analysis of recombinant TonB at OD 450 nm using the different clinical and commercialized serums at 1:100 concentration. The results are expressed as optical density (OD). Each experiment was performed in triplicates. The normal human and rabbit sera were responsible for the negative control.....	229
Figure 4.28	The weekly mean body weights of the control group, OMPs-immunized, and SAPs-immunized rats from days 0 to 42. Each test group was administered 100 µg protein by intraperitoneal route as the first immunization on test day 0. On days 14 and 28, the rats were injected subcutaneously with 100 µg of the respective protein. The control group was injected with PBS in each immunization session. Each rat was sacrificed on day 42. Each data point represents the mean body weight ± standard deviation.	231
Figure 4.29	Immunoblot analysis of serum pooled from rats immunized with OMPs. Serum IgG recognized the OMPs after the (a) first immunization and enhanced after the (b) second and (c) third immunization with OMPs. M: marker (kDa); C: control.....	233
Figure 4.30	IgG response following immunization of Sprague Dawley rats with OMPs from <i>S. flexneri</i> (ATCC12022) by intraperitoneal route during the first immunization and subcutaneous route for the subsequent immunization. The antibody was determined by ELISA (antibody capture) using 1:100 serum collected from the rats from pre-immunization until third immunization. A <i>p</i> value of less than 0.05 was considered statistically significant versus the control group. * <i>p</i> < 0.05; ** <i>p</i> < 0.01; *** <i>p</i> < 0.001. Error bars represent standard deviation.....	234
Figure 4.31	Immunoblot analysis of serum pooled from rats immunized with SAPs. Serum IgG recognized the SAPs after the (a) first	

- immunization and enhanced after the (b) second and (c) third immunization with SAPs. M: marker (kDa); C: control.....236
- Figure 4.32 IgG response following immunization of Sprague Dawley rats with SAPs from *S. flexneri* (ATCC12022) by intraperitoneal route during the first immunization and subcutaneous route for the subsequent immunization. The antibody was determined by ELISA (antibody capture) using 1:100 serum collected from the rats from pre-immunization until third immunization. A *p* value of less than 0.05 was considered statistically significant versus the control group. * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001. Error bars represent standard deviation.....237
- Figure 4.33 Gel electrophoresis of RNA of spleen isolated from the immunized Sprague Dawley with OMPs and SAPs, individually. The RNAs from the control group, OMP-immunized group, and SAPs-immunized group were run on 1% agarose gel at 120 V for 45 minutes. The intense bands were 28s ribosomal RNA, and the less intense bands at the bottom were 18S ribosomal RNA. R1: Rat 1; R2: Rat2; R3:Rat 3.....240
- Figure 4.34 The bar chart shows the gene expression of (a) TNF α , (b) IL-1 β , (c) IL-6, and (d) IL-12 by groups of rats that were immunized with 100 mg of OMPs and SAPs from *S. flexneri*, respectively. qPCR was performed at 40 cycles, and the Ct values of each gene level were normalized with GAPDH. The presented data are the mean and standard deviation from biological and technical triplicates. Statistical analysis was done by two-way ANOVA. A *p* value of less than 0.05 was considered statistically significant. * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001. Error bars represent standard deviation.242
- Figure 4.35 The bar chart shows the gene expression of IL-10 by groups of rats immunized individually with 100 mg of OMPs and SAPs from *S. flexneri*. qPCR was performed for 40 cycles, and the Ct values from each sample were compared with GAPDH. The presented

	data are the mean and standard deviation from biological and technical triplicates. Statistical analysis was done by two-way ANOVA. A p value of less than 0.05 was considered statistically significant. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Error bars represent standard deviation.....	244
Figure 4.36	Immunoblot analysis to determine the reactivity of the recombinant FepA with the OMPs-immunized rat serum IgG and SAP-immunized rat serum IgG. M: marker (kDa); C: control.	246
Figure 4.37	ELISA analysis of recombinant FepA against the OMP-immunized rat serum and SAP-immunized rat serums at 1:100 concentration. The results are expressed as optical density (OD). Each experiment was performed in triplicates. The normal human and rabbit sera were responsible for the negative control. Statistical analysis was done by two-way ANOVA. A p value of less than 0.05 was considered statistically significant. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Error bars represent standard deviation.	248
Figure 4.38	Immunoblot analysis to determine the reactivity of the recombinant TonB with the OMPs-immunized rat serum IgG and SAP-immunized rat serum IgG. M: marker (kDa); C: control.	250
Figure 4.39	ELISA analysis of recombinant TonB at OD 450 nm using the different clinical and commercialized serums at 1:100 concentration. The results are expressed as optical density (OD). Each experiment was performed in triplicates. The normal human and rabbit sera were responsible for the negative control. Statistical analysis was done by two-way ANOVA. A p value of less than 0.05 was considered statistically significant. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Error bars represent standard deviation.	252
Figure 5.1	The pET28a plasmid vector. The vector contains an origin of replication (<i>ori</i>), an antibiotic resistance gene (<i>Kan</i>), a promoter, ribosome binding site (RBS), N-terminal tag, multiple cloning site (MCS), and terminator. Image provided by Novagen.....	281

LIST OF SYMBOLS

°C	Degree Celsius
Δ	Delta
μ	Micro
™	Trademark
®	Registered
×	Multiplication
× g	Relative centrifugal force
β	Beta
α	Alpha
γ	Gamma
±	Plus-minus
=	Equal to
<	Less than
amu	Atomic mass unit
CFU/mL	Colony forming unit per milliliter
μL	Microliter
μM	Micromolar
KDa	Kilo dalton
L	Liter
mL	Milliliter
mM	Milimolar
nm	Nanometer
rpm	Revolutions per minute

LIST OF ABBREVIATIONS

APCs	Antigen-presenting cells
BLASTp	Protein basic local alignment search tool
BSC	Biosafety cabinet
CD4+	Cluster of differentiation 4
CD8+	Cluster of differentiation 8
cDNA	Complementary deoxyribonucleic acid
CIP	Calf intestine phosphatase
CO ₂	Carbon dioxide
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leukocyte antigen
IC ₅₀	Half maximal inhibitory concentration
IgG	Immunoglobulin G
IL-1 β	Interleukin-1 beta
IL-6	Interleukin-6
IL-12	Interleukin-12
INF- γ	Gamma interferon
iNOS	Inducible nitric oxide synthase
Ipa	Invasion plasmid antigen
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
M cells	Microfold cells
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NaOH	Sodium hydroxide

NCBI	National Center for Biotechnology Information
NO	Nitric oxide
OMPs	Outer membrane proteins
ORF	Open reading frame
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PMA	Phorbol 12-myristate-13-acetate
PMN	Polymorphonuclear
PMSF	Phenylmethylsulfonyl fluoride
PRRs	Pattern-recognition receptors
RT-qPCR	Real time-quantitative polymerase chain reaction
RPMI	Roswell park memorial institute medium
SAPs	Surface-associated proteins
<i>S. boydii</i>	<i>Shigella boydii</i>
<i>S. dysenteriae</i>	<i>Shigella dysenteriae</i>
<i>S. flexneri</i>	<i>Shigella flexneri</i>
<i>S. sonnei</i>	<i>Shigella sonnei</i>
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH057	Virulent strain of <i>Shigella flexneri</i> 2a
SH062	Mild strain of <i>Shigella flexneri</i> 2a
SS	<i>Salmonella-Shigella</i>
TAE	Tris-acetate-EDTA
TFA	Trifluoroacetic acid
TNF- α	Tumor necrosis factor-alpha
THP-1	Human monocytic cell line derived from an acute monocytic leukemia patient
TLR	Toll-like receptors
V	Volts

LIST OF APPENDICES

Appendix A	Materials, reagents, cells, phages, instruments, and software
Appendix B	Ethical approvals
Appendix C	List of publications
Appendix D	List of oral presentations

**PENEROKAAN PROFIL IMUNOMODULASI TITISAN SEL
TERBITAN MAKROFAJ MANUSIA THP-1 YANG DIPERANTARAKAN
OLEH *Shigella flexneri***

ABSTRAK

Shigellosis adalah punca utama cirit-birit yang teruk, terutamanya dalam kalangan kanak-kanak berumur kurang daripada lima tahun. Kejadian strain tahan pelbagai ubat spesies *Shigella* telah menyumbang kepada ketidakberkesanan rawatan sedia ada. Vaksinasi telah menjadi keperluan penting untuk mencegah shigellosis. Penciptaan vaksin berasaskan epitope telah mengarahkan pembangunan model reka bentuk vaksin baharu. Makrofaj ialah APC khusus yang memproses antigen diikuti dengan mempersembahkan antigen yang diproses kepada sel-T melalui molekul HLA. Profil imunomodulator makrofaj yang dijangkiti oleh strain klinikal *S. flexneri* 2a kekal tidak ditentukan. Makrofaj terbitan THP-1, sebagai model makrofaj manusia, telah dijangkiti secara bebas dengan strain ringan dan virulen *S. flexneri* 2a pada keadaan pertumbuhan standard. Tahap ekspresi gen mediator inflamasi dikenal pasti menggunakan qPCR, manakala pengeluaran NO diukur menggunakan kit ujian NO yang dikomersialkan. Regulasi tinggi TNF α , IL-1 β , IL-6, IL-12, iNOS dan NO yang ketara menunjukkan tindak balas pro-radang terhadap jangkitan *S. flexneri* 2a. Ketegangan virulen juga menyebabkan peningkatan ketara dalam tahap ekspresi mRNA sitokin anti-radang. Ekspresi gen kelas II HLA juga disiasat untuk melanjutkan kajian imunopeptidomik dan mendapati pengawalseliaan ketara kelas II HLA oleh makrofaj yang dijangkiti *Shigella*. Strategi imunopeptidomik berasaskan LC-MS/MS digunakan untuk mengenal pasti peptida yang boleh digunakan sebagai calon berpotensi untuk pembangunan vaksin *Shigella* berasaskan epitope. Makrofaj terbitan

THP-1 telah dijangkiti secara individu dengan strain ringan dan virulen *S. flexneri* 2a. Kompleks kelas II HLA-peptida telah diimmunopresipitasi dengan anti-HLA DR, DP, dan DQ dan dianalisis menggunakan LC-MS/MS. Penempatan jujukan telah diramalkan menggunakan tiga alat bioinformatik dan mendapati 14 peptida sebagai OMP. Selepas dianalisis menggunakan beberapa alat bioinformatik, serpihan daripada reseptor membran luar FepA dan reseptor yang bergantung kepada TonB diramalkan sebagai calon vaksin *Shigella* yang berpotensi. Rekombinan protein terpilih telah dibina untuk ujian imunogenisiti. Serum tikus yang dijangkiti *Shigella* diperoleh dengan mengimunkan tikus secara bebas dengan OMP dan SAP *S. flexneri*. Ujian imunogenisiti dilakukan dengan mengesan antibodi daripada serum tikus yang diimmunisasi menggunakan protein rekombinan yang dibina oleh imunoblot dan ELISA. FepA dan TonB rekombinan adalah reaktif dengan serum tikus imunisasi OMP dan SAP. Kesimpulannya, FepA dan TonB rekombinan adalah calon yang sangat berpotensi untuk pembangunan vaksin *Shigella* berasaskan epitope, dan keberkesanannya boleh dinilai lebih lanjut dalam kajian imunisasi haiwan.

**EXPLORING THE IMMUNOMODULATION PROFILES OF THE
THP-1 HUMAN MACROPHAGE-DERIVED CELL LINE MEDIATED BY
*Shigella flexneri***

ABSTRACT

Shigellosis is the primary cause of severe diarrhea, especially among children less than five years old. The occurrence of multi-drug resistant strains of *Shigella* species has contributed to the ineffectiveness of the existing treatment. Vaccination has become an essential requirement for preventing shigellosis. The invention of an epitope-based vaccine has directed the development of a new vaccine design model. Macrophages are specialized APCs that process antigens and then present the processed antigens to T-cells through HLA molecules. The immunomodulatory profiles of the macrophages infected by the clinical strains of *S. flexneri* 2a have remained undefined. THP-1-derived macrophages, as the model of human macrophages, were infected independently with mild and virulent strains of *S. flexneri* 2a at standard growth conditions. The gene expression level of inflammatory mediators was determined using qPCR, while NO production was measured using a commercial NO assay kit. The significant up-regulation of TNF α , IL-1 β , IL-6, IL-12, iNOS, and NO indicates the pro-inflammatory reaction to *S. flexneri* 2a infection. The virulent strain also markedly increased the expression levels of the anti-inflammatory cytokine mRNAs. HLA class II gene expression was also investigated to extend the immunopeptidomics study and found the significant up-regulation of HLA class II by the *Shigella*-infected macrophages. LC-MS/MS-based immunopeptidomics strategy was employed to identify the peptides that can be used as potential candidates for the epitope-based *Shigella* vaccine development. THP-1-derived macrophages were

individually infected with the mild and virulent strains of *S. flexneri* 2a. The HLA class II-peptide complexes were immunoprecipitated with anti-HLA DR, DP, and DQ and analyzed using LC-MS/MS. The localization of the sequences was predicted using three bioinformatic tools and found 14 peptides as OMPs. After being analyzed using several bioinformatics tools, fragments from outer membrane receptor FepA and TonB-dependent receptors were predicted as the potential *Shigella* vaccine candidates. The recombinant of the selected proteins was constructed for immunogenicity assay. *Shigella*-infected rat serum was obtained by independently immunizing rats with OMPs and SAPs of *S. flexneri*. The immunogenicity assay was performed by capturing the antibody from the serum of the immunized rat using the constructed recombinant proteins by immunoblot and ELISA. The recombinant FepA and TonB were reactive with the OMPs- and SAPs-immunized rat serum. In conclusion, recombinant FepA and TonB were highly potential candidates for epitope-based *Shigella* vaccine development, and the efficacy can be further evaluated in animal immunization studies.

CHAPTER 1

INTRODUCTION

This thesis provides six chapters and displays the second and third sub-chapter. The thesis starts with the introduction in Chapter 1, which outlines the overall thesis content and presents an overview of the knowledge and the area of the knowledge gap. The research questions and objectives of the study are clearly stated to ensure the direction of content in this thesis is focused on the area of interest.

1.1 Background

Diarrhea is a global human health issue. Although usually assumed as a less critical health problem, several studies focus on the significance of diarrhea as a continuing factor of mortality among children, adolescents, and adults in developing countries (Lamberti et al., 2012). While child mortality rates have been reduced in the previous few decades, diarrhea is still the second most common factor of death among children under five years old, which causes 1 in 11 death worldwide (Liu et al., 2015).

Shigella is a bacterial pathogen that contributes significantly to the global burden of diarrheal disease (Zaidi & Estrada-García, 2014). A study in seven developing countries in sub-Saharan Africa and South Asia identified *Shigella* as the primary cause of diarrheal disease among children under five years old (Kotloff et al., 2013). Another study conducted in eight countries in sub-Saharan Africa, South America, and South Asia found that *Shigella* was one of four pathogens related to the leading burden of diarrheal diseases during two years old (Platts-Mills et al., 2015). Nine-year research on diarrhea in the Northern region of Malaysia found the *Shigella* species is the third most common bacterial pathogen that can cause diarrhea among children in this region (BangaSingh et al., 2011).

Shigella is an etiological agent for shigellosis. It is a rod-shaped, Gram-negative bacteria and facultative enteric intracellular pathogen. Kiyoshi Shiga, a scientist from Japan, reported the first isolation of *Shigella* in 1897. Now, after more than 120 years of discovery, shigellosis is still a critical public health issue, particularly in unsafe water supplies and poor hygiene (Niyogi, 2005). Every year up to 165 million cases of shigellosis worldwide, of which around one million deaths are reported among children under five years old (Killackey et al., 2016). Besides affecting young children, *Shigella* also targets the elderly, immunocompromised, and malnutrition persons (Khan et al., 2013).

Shigellosis is an acute intestinal disease spread from person to person through the fecal-oral route by ingesting contaminated food or water. The ingestion of 10 to 100 bacilli is enough to cause severe shigellosis (DuPont et al., 1989). *Shigella* can survive in acidic conditions in the host stomach, partially related to the low infection dose of this bacteria (Gorden & Small, 1993b). *Shigella* can also induce the reduction of antibacterial peptides secreted continuously from the mucosal membrane of the gastrointestinal tract (Islam et al., 2001). The clinical manifestation of shigellosis includes vomiting, abdominal cramp, fever, and diarrhea with mucous and blood. The untreated shigellosis can precede more severe illness and long-term effects such as reactive arthritis and kidney failure (Burnett, 2017). Therefore, effective management and prevention are required to control shigellosis incidence long-term.

Prevention and management of shigellosis have currently become a significant public health challenge. Presently, the occurrence of the multi-drug resistance of *Shigella* makes the treatment of shigellosis increasingly difficult and challenging (Puzari et al., 2018). A new initiative to treat and prevent shigellosis is highly essential. Experiments proved that *Shigella* infection gives protective immunity. In the endemic

areas, shigellosis incidences are high among children under five years old and reduced after that age, indicating that immunity developed after repeated exposure to shigellosis during the first five years (Taylor et al., 1986). Compelling evidence shows the primary infection of *Shigella* presented 76% protective efficacy in response to the reinfection with the same serotype (Ferrecchio et al., 1991). This finding indicates the *Shigella* vaccine could be a practical approach that significantly reduces the disease burden.

To date, several *Shigella* vaccines have been developed, including the killed bacteria, live-attenuated, and epitope-based vaccines. Unfortunately, no *Shigella* vaccine has so far been licensed (Zaidi & Estrada-García, 2014). The existing *Shigella* vaccines lack the right balance of robust immunogenicity and are not sufficiently attenuated (Ashkenazi & Cohen, 2013). The idea of forming a vaccine from selective few epitopes has emerged as a more logical approach since the conventional approaches need a longer time to be produced, and the selection of antigens is more or less random. Identifying immunogenic epitopes from *Shigella* is another choice for developing effective epitope-based *Shigella* vaccines.

The interaction between antigen-presenting cells (APCs) and T-cells is considered a critical collaboration between the innate and adaptive arms of the immunity. Both immune responses are necessary to defend the host from the *Shigella* infection (Toapanta et al., 2018). Macrophages are the APCs mainly known for triggering host defense by engulfing foreign particles, degrading the particles, and presenting small parts of the foreign peptide on major histocompatibility complex (MHC) molecules to T-cell receptors to activate adaptive immune response (Neefjes et al., 2011). The activation of the cell-mediated immunity primarily against any

foreign or non-self antigens relies on identifying epitopes or peptides presented on the HLA of the APCs (Mellman & Steinman, 2001).

Knowledge about *Shigella*-derived molecules or epitopes that induce T-cell-specific responses is critical to understand the protection against shigellosis and developing further effective vaccines. Outer membrane proteins (OMPs) of *Shigella* are appropriate immunogenic proteins and effectively induce the immune response of the host (Pore et al., 2011). Identifying specific and antigenic OMPs in *Shigella* is essential to determine the suitable candidate for the *Shigella* vaccine development. The mass spectrometry-based immunopeptidomics approach offers a method to identify peptide sequences derived from the OMPs of *Shigella* presented by HLA class II of *Shigella*-infected macrophages. The identified immunopeptidomes are a highly versatile group of peptides presented on the surface of the macrophages to T-cell receptors, which after their recognition, activate T-cells.

Understanding the immunomodulatory mechanism of macrophages mediated by *Shigella* is crucial for disease prevention and therapeutic intervention. The present study investigates the immunomodulation roles of activated macrophages mediated by *Shigella*. The study will also explore the processed peptides presented by *Shigella*-infected macrophages. The peptides signify the epitopes that are naturally processed and present in a form identifiable by the immune system of the host.

1.2 Problem statement

Shigella is among the most common pathogens accountable for the burden of diarrheal illness and death among children under five years old. Antibiotic treatment is recommended to reduce disease severity. However, due to the risk of antibiotic resistance, shigellosis cannot be continuously treated by mass drug administration. The

prevention of shigellosis would primarily depend on developing a vaccine to strengthen the immune system and fight this microbial pathogen. Before developing the vaccine, a comprehensive understanding of the shigellosis-associated immune response is critically essential. Developing an effective vaccine that induces T-cell-specific responses may provide long-lasting memory and reliable immunity against shigellosis. In the present study, acquiring valuable knowledge about immunomodulatory roles of the human macrophages and peptides presentation by activated macrophages-mediated *Shigella* is crucial for future immunotherapy development. The peptides are potentially the candidate for developing the epitope-based *Shigella* vaccine.

In the present study, *S. flexneri* 2a mild (SH062) and virulent (SH057) strains isolated from Malaysian patients are the strain of choice instead of the commercial reference bacteria. The use of clinical strains gives a similar expression as the real infection and the proteins presented by the HLA molecules of macrophages derived from the isolated strains. These strains were previously determined for the protection efficacy and the OMPs expression conditions (BangaSingh et al., 2016; Harikrishnan et al., 2013). The differences between both clinical strains of *S. flexneri* 2a were previously investigated by BangaSingh et al. (2016). The virulent strain is the wild type of *S. flexneri* 2a and showed resistance to tetracycline (BangaSingh et al., 2016). Sereny test was performed to study the ability of both clinical strains to invade and multiply within the corneal epithelium of guinea pig, as the experiment closely mimics the invasion process in the intestinal epithelium (Powell et al., 1991). The virulent strain showed more virulence in all the guinea pig eyes tested than the mild strain in this test.

The immunomodulation profiles of the human macrophages mediated by the clinically isolated mild and virulence strains of *S. flexneri* 2a need to be identified. Data from this study can be used to study the heterologous protection of these strains against other virulent *Shigella* species.

1.3 Research questions

- i. What are the gene expression profiles of inflammatory mediators secreted by macrophages in response to *Shigella* infection?
- ii. What peptides are bound to HLA class II of macrophages upon *Shigella* infection?
- iii. What are the immunoreactivity profiles of the rats immunized with OMPs and SAPs of *S. flexneri*?
- iv. What is the immunogenicity status of the selected immunopeptidomes from the *Shigella*-infected macrophages?

1.4 Objectives

1.4.1 General objective

To investigate the immunomodulatory profile of macrophages in response to *Shigella* infection, which leads to the identification of immunogenic proteins presented by the HLA class II of macrophages.

1.4.2 Specific objectives

- i. To identify the gene expression profiles of inflammatory mediators secreted by *Shigella*-infected macrophages.

- ii. To investigate the peptides bound to HLA class II of macrophages upon *Shigella* infection.
- iii. To determine the immunoreactivity profiles of rats immunized with OMPs and SAPs of *Shigella*.
- iv. To identify the immunogenicity status of selected immunopeptidomes from *Shigella*-infected macrophages.

1.5 Significance of the study

Shigella infection exemplifies an exciting model for the immune response and inflammation imbalance. These bacteria also developed several strategies to escape from killing by the immune cells of the host. The antimicrobial peptides expressed in phagocytic cells and at epithelial surfaces are the effector molecules of innate immunity. However, it has been reported that *Shigella* can turn off the expression of antimicrobial peptides, subsequently resulting in severe shigellosis. Like many other tropical diseases, treatment with an antibiotic is considered to manage shigellosis.

Nevertheless, the existence of multi-drug resistance of *Shigella* strains complicates the treatment, causing the prevention of infection is essential. Although the vaccine is often known to offer excellent protection in the host, there is still a lack of appropriate vaccines that can be used to prevent shigellosis. Therefore, in acquiring valuable knowledge for developing the epitope-based *Shigella* vaccine, the discovery of peptide presentation by activated macrophages mediated *Shigella* is increasingly required to reduce the worldwide impact of shigellosis.

1.6 The layout of the thesis

The following chapters describe the details of this study. In chapter 2, the gap in the knowledge discussed in Chapter 1 is explicitly explained through a literature review. The content connects the global incidences of shigellosis, which leads to the need to develop a *Shigella* vaccine. The theoretical background about shigellosis is briefly outlined, and the past work relevant to the host immune response against *Shigella* infection was briefly explained in this chapter. A clear hypothesis about the accountability of macrophages in engulfing pathogen and presenting the peptide to the T-cells strengthens the need to use these cells to identify the peptides as candidates for the development of epitope-based *Shigella* vaccine.

The methods employed to respond to the research questions and to test the hypothesis of the study was elaborated in Chapter 3. This chapter mentions every step involved in conducting this research. The chapter presented a figure illustrating the summary of the overall procedure to explore the research questions. The preparations of reagents, buffers, and media are included in this chapter. A detailed explanation of the research methodology and the data analysis techniques are outlined. The collected data are displayed in chapter 4. The raw data are not presented in this chapter, but the way to collect, process, and analyze the data are briefly described. The data are presented in the bar chart, pie chart, graph, and table. The results are subsequently appraised, and explanations are suggested for particular outcomes in the data.

Chapter 5 discusses the central results and the potential implications of the study. The results are interpreted, and the theoretical and clinically relevant implications of the findings are also considered. In this chapter, the choice of methods is also discussed, in which the possible influence of methodological biases and errors

on data validity. The result discussions are also connected to the theory outlined in the literature review forming the basis of the conclusions.

Chapter 6, the final chapter, summarizes the answer to the research questions, and conclusions are drawn. A strong statement is made in this chapter based on the finding. The content addresses the strengths and general limitations of the study. The proposed areas for future research are also recommended in this chapter.

CHAPTER 2

LITERATURE REVIEW

2.1 A general overview of *Shigella* and shigellosis

In developing countries, infectious diarrhea is the second most common cause of death among children under five years old (Walker et al., 2012; Liu et al., 2015). Diarrhea is generally understood to describe the passage of usually watery or loose feces at least three times per day (Hellard et al., 2003). Every year, nearly 1.7 billion cases of childhood diarrhea are reported by the World Health Organization globally, with an estimated 525,000 deaths yearly. These deaths occur primarily in South Asia and Africa (“WHO. Factsheet on Diarrhoeal disease,” 2017).

Numerous studies aimed to determine the species of pathogens in diarrheal stools. With *Salmonella* species and *Entamoeba histolytica*, *Shigella* species are among the primary pathogens of diarrhea isolated from patients in many countries (Walker, Sack, and Black, 2010). A research finding by Zaidi and Estrada-García (2014) also points to *Shigella* as one of the most common causes of diarrheal illness among children, significantly contributing to the burden worldwide.

2.1.1 *Shigella*

Shigella bacteria are Gram-negative bacillus, non-motile, non-spore-forming, non-lactose fermenter, and facultative anaerobes that belong to the Enterobacteriaceae family. The morphology of *Shigella* on the *Salmonella-Shigella* (SS) agar plate and under the light microscope is showed in Figure 2.1. This bacterium shares many common properties with *Escherichia coli* (*E. coli*) strains, and their genetic similarity strongly suggests that they represent a subtype of *E. coli* (Mattock & Blocker, 2017).

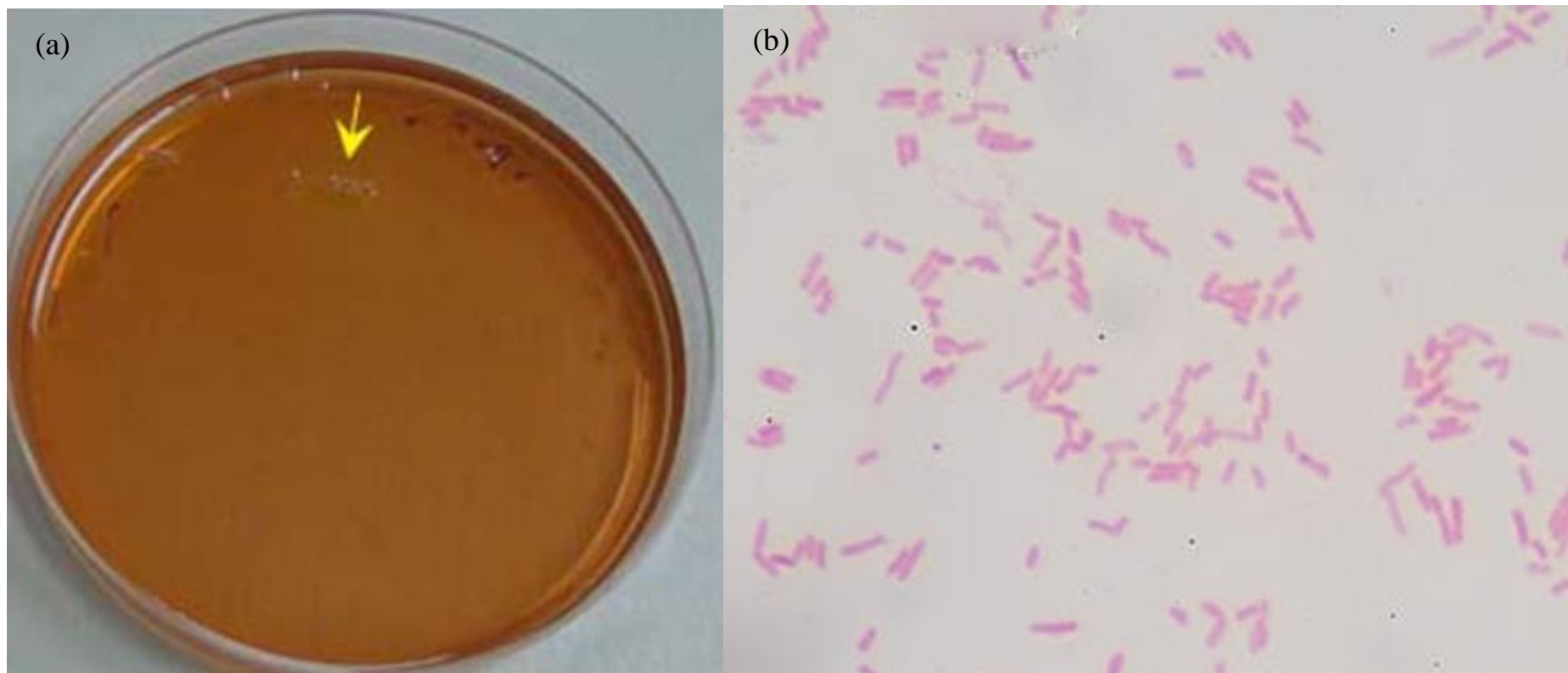


Figure 2.1: (a) The morphology of *Shigella* colonies on SS agar plate. The colonies showed smooth, colorless, without black-centered. (b) *Shigella* was stained with Gram's stain and observed under the light microscope with a 40× objective lens. The shape of the bacteria is rod-shaped and stained with pink color, indicating the Gram-negative bacteria.

With the introduction of enteroinvasive *E. coli*, the distinction between *Shigella* and *E. coli* has become even more difficult. Enteroinvasive *E. coli* are strains that exhibit some of the biochemical properties of *E. coli* and can produce dysentery utilizing the same invasion mechanism as *Shigella*. Enteroinvasive *E. coli* is more closely linked to *Shigella* than non-invasive *E. coli*, according to the sequencing of numerous housekeeping genes (Ragupathi et al., 2018). Within *E. coli*, *Shigella* and enteroinvasive *E. coli* originated from the same origin and formed a single pathovar. A polymerase chain reaction (PCR) targeting the ipaH-gene, a multicopy gene present solely in *Shigella* and enteroinvasive *E. coli*, could be used to distinguish *Shigella* and enteroinvasive *E. coli* from other *E. coli* (Van Den Beld & Reubsæet, 2012).

Shigella is classified into four species from more than forty *Shigella* serotypes. The four *Shigella* species are *Shigella flexneri* (*S. flexneri*; 19 serotypes and two variances, serogroup B), *Shigella sonnei* (*S. sonnei*; one serotype, serogroup D), *Shigella dysenteriae* (*S. dysenteriae*; 15 serotypes, serogroup A), and *Shigella boydii* (*S. boydii*; 20 serotypes, serogroup C) (Asbury et al., 2018). *Shigella* are divided into different serotypes based on their O antigen molecule of lipopolysaccharide (LPS) found in the outer membrane (Mattock & Blocker, 2017). LPS is one of the major components of the outer membrane of Gram-negative bacteria, a complex molecule consisting of a lipid A anchor, a polysaccharide core, and chains of carbohydrates. Sugars in the polysaccharide chains confer serologic specificity. The *S. flexneri*, *S. dysenteriae*, and *S. boydii* are physiologically similar, while *S. sonnei* differs from the other species based on biochemical reactions. The endemic disease is mainly caused by *S. flexneri* and *S. sonnei*, while the epidemic disease is commonly due to *S. dysenteriae* (Niyogi, 2005).

2.1.2 Shigellosis incidences worldwide

Children under five years old are vulnerable to *Shigella* infection, and this age group is the top proportion of shigellosis and death incidence (Asbury et al., 2018). However, during the first six months of life, shigellosis is rare due to the relatively low direct interaction with the environment and the presence of maternal immunity. After the six months, the incidence increases, peaking at 12 to 23 months and decreasing moderately afterward (Kotloff et al., 2013).

The PCR data show a higher *Shigella* disease burden than the conventionally practiced estimate (Livio et al., 2014). A study that isolates *Shigella* from feces and food samples by conventional culture methods and PCR proves the utilization of the PCR method enhanced the identification rate of *Shigella* in food samples from 2.1% to 8.6% and feces samples from 6.7% to 14% (Mokhtari et al., 2012). The other study that diagnoses shigellosis using the quantitative polymerase chain reaction (qPCR) found that the rate of diarrhea cases related to *Shigella* almost doubled, from 9.6% by conventional culture methods to 17.6% by qPCR (Lindsay et al., 2013). This finding confirms that conventional culture methods may underrate the global burden of shigellosis.

Shigella strains have similar pathogenic properties, unique epidemiological characteristics, and varied dissemination arrangements in different geographical regions (Puzari et al., 2018). Shigellosis incidences are mainly in Africa, Asia, and Latin America, where limited access to clean water and poor hygiene promotes the transmission of *Shigella* (Bennish & Ahmed, 2020). Particular *Shigella* species are more dominant than the others in a particular region of the world, as shown in Figure 2.2.

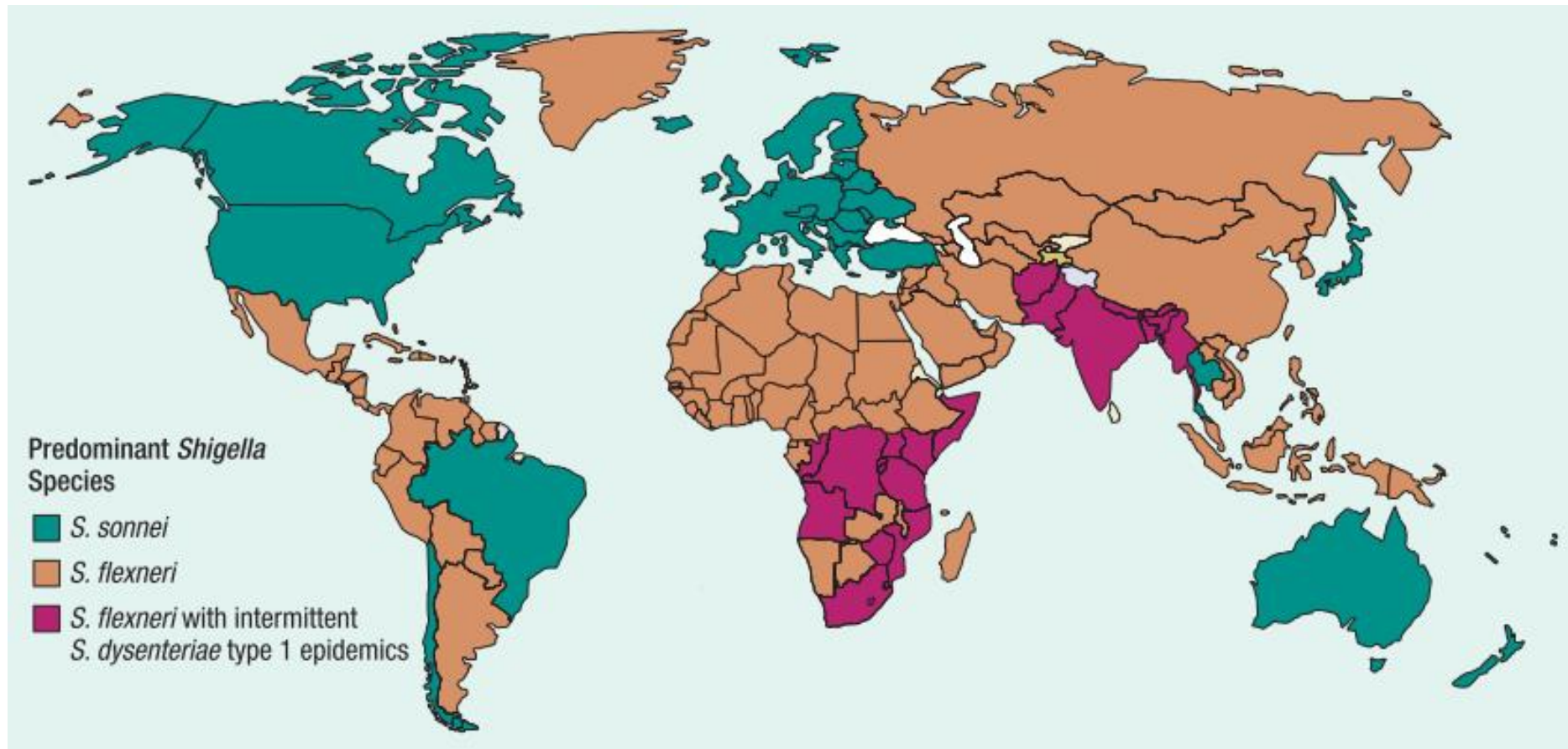


Figure 2.2: The global distribution of *Shigella* species. Shigellosis caused by *S. boydii* is not displayed because it is uncommon. Data source: (Bennish & Ahmed, 2020).

Among the *Shigella* species, *S. flexneri* is the most predominant species in the endemic and developing countries (Lin, 2002; Lee and Puthuchery, 2003; Singh *et al.*, 2011). *S. flexneri* 2a and *S. dysenteriae* 1 is the most commonly isolated species of *Shigella* in Asia and Africa, whereas *S. sonnei* is the most commonly isolated species in developed countries, including Europe and the United States (Salam & Bennish, 1991). *S. dysenteriae* is accountable for epidemic dysentery in the developing world. The fourth species, *S. boydii*, accounts for 6% or less of *Shigella* infections (Kotloff *et al.*, 1999).

In developing countries, the distribution percentage of *S. flexneri*, *S. sonnei*, *S. boydii*, and *S. dysenteriae* is 60%, 15%, 6%, and 6%, respectively (Kotloff *et al.*, 1999). The most notable *Shigella* species for developing vaccines are *S. flexneri* 2a, *S. dysenteriae* 1, and *S. sonnei* (Sansonetti, 2008). Bennish and Wojtyniak (1991) explain that *S. flexneri* 2a causes more mortality rate than the other species of *Shigella* in the developing world. Kotloff *et al.* (1999) found a similar finding in the study that reported *S. flexneri* 2a is the predominant serotype of *S. flexneri*. This serotype is the most prevalent species that causes bacillary dysentery or shigellosis.

2.1.3 Shigellosis incidences in Malaysia

In Malaysia, floods are regular natural disasters. It takes place during the monsoon season almost every year. Figure 2.3 shows the Northeast monsoon from October to March and the Southwest monsoon from May to September yearly (Diya *et al.*, 2014). The East Coast of the Peninsular Malaysia and parts of Sabah and Sarawak are the most affected places during the monsoon season (Bamaiyi *et al.*, 2016). Besides causing life loss and property damage, floodwater is biologically responsible for water contamination (Khan *et al.*, 2014).



Figure 2.3: The map shows the monsoons that hit Malaysia every year. Southwest monsoon (.....→) arises from May to September, and Northeast monsoon (—→) occurs from October to March. The figure was created with BioRender.com.

The floodwater is a rich source of various microorganism species with different degrees of antibiotic resistance (D/iya et al., 2014). *Shigella* is a type of bacteria transported in the floodwater from one place to another during the flood (Bamaiyi et al., 2016). Floodwater sampling from 15th December 2014 until 3rd January 2015 showed the presence of *S. flexneri* in the flood areas along the East Coast of Peninsular Malaysia (Bamaiyi et al., 2016; Basri et al., 2015). The microbiological evaluation of floodwater in this region is consistent with the finding in Thailand, the north of Peninsular Malaysia. During the flood in Thailand in 2011, the presence of *Shigella* species in floodwater and tap water was reported (Chaturongkasumrit et al., 2013).

Nine-year research on shigellosis in the Northern region of Malaysia found that the *Shigella* species is the third leading bacterial agent that can cause diarrhea in children (BangaSingh et al., 2011). Isolates of *Shigella* species in Malaysia show resistance to multiple types of drugs. Seventy-three percent out of a hundred samples of *Shigella* isolated in Malaysia showed resistance to at least one antibiotic (Lin et al., 2002). Most *Shigella* species were resistant to kanamycin, trimethoprim-sulphamethoxazole, trimethoprim, chloramphenicol, ampicillin, tetracycline, and streptomycin (BangaSingh et al., 2011; Lin et al., 2002). The high prevalence of multi-drug-resistant *Shigella* and the high incidence of shigellosis during the flood event in Malaysia indicate the need for strategic control against the infection of this disease. Besides improving public hygiene and avoiding the excessive use of current antimicrobial agents, developing a vaccine is an excellent alternative to prevent shigellosis.

2.1.4 Transmission of *Shigella* and clinical manifestation of shigellosis

Shigellosis is associated with malnutrition and poor hygienic standards. This disease is a highly infectious enteric bacterial disease. An infected person who

experiences diarrhea is primarily responsible for transmitting shigellosis (Levine and Levine, 1991). There is no recognized reservoir for *Shigella*. The infection is spread from person to person, most commonly through fecal-oral contamination (Carayol and Nhieu, 2013). The ingestion of contaminated water and food is the common cause of *Shigella* infection (Asbury et al., 2018). A study by Levine and Levine (1991) reported a relationship between the transmission of *Shigella* infection to humans through fly vectors. The flies land on human excrement, which contains bacterium, followed by delivering the disease to fomites such as a dish or doorknob that, in turn, leads to social inoculation.

The transmission control of *Shigella* is difficult as the ingestion of as few as 10 to 100 bacilli is sufficient to cause dehydration caused by severe diarrhea (Schmid-Hempel & Frank, 2007). This low infectious dose affects the disease to spread quickly (Sethuvel et al., 2017). The incubation period depends on the infecting strains, ranging from 6 to 96 hours. No group of individuals is immune to the *Shigella* infection, but a specific person is at higher risk. In a healthy person, the infection is self-limiting, but in malnourished children or immunocompromised individuals, the illness stays for several days to weeks or even months (Kotloff et al., 2013).

The invasion of *Shigella* to the human intestine affects a spectrum of clinical presentations and begins with symptoms such as malaise, fatigue, fever, and short-lasting watery diarrhea. Shigellosis can precede illness from mild to severe diarrhea with the recurrent passage of mucus and blood, abdominal cramping and pain, nausea, high fever, chills, vomiting, and in a few cases, bacteremia (Replogle et al., 2000). The range of clinical symptoms of shigellosis among the elderly above 65 is as severe as in children (Casburn-Jones & Farthing, 2004). Diarrhea can cause the body to dehydrate and lack the salts necessary for survival (Carayol & Nhieu, 2013). The die

incidence of diarrhea has resulted chiefly from severe dehydration and fluid loss (WHO, 2013).

2.1.5 The emergence of multi-drug resistant *Shigella*

Healthy individuals with mild *Shigella* infection usually recover with oral rehydration and home remedies. Dehydration due to diarrhea of any etiology could be corrected by rehydration therapy, and this treatment has dramatically decreased the number of death caused by diarrhea (Niyogi, 2005). However, *Shigella* that invades the intestinal epithelium, which responds to moderate or severe shigellosis, needs special treatment to cure. Antimicrobial therapy is an effective treatment to recover shigellosis by decreasing the shedding of organisms, reducing the severity, and preventing the possibility of lethal complications (Sur et al., 2004). Without antimicrobial treatment or an ineffective antimicrobial is given, an episode of shigellosis lasts from two to 10 days or longer. The risk of serious complications or death increases significantly (Niyogi, 2005).

However, antimicrobial agents become less effective since most pathogens attempt to obviate antibiotics which cause harm and death to the microorganisms (Dhama et al., 2015). This condition results in the increasing failure rates of treatment for bacterial infectious disease as the antibacterial agent is no longer effective against the bacteria (Puzari et al., 2018). The primary mechanism of drug resistance bacteria might be drug modification, alteration of a metabolic pathway, alteration of the target site increasing the active drug efflux across the cell surface, or decreasing drug permeability resulting in reduced drug accumulation (Blair et al., 2015).

Shigella species are included in multi-drug-resistant bacteria in developing countries (Pazhani et al., 2008). *Shigella* showed resistance to primarily used antimicrobial agents for a previous couple of decades. The drug choices become

limited because of the global occurrence of multi-drug-resistant of *Shigella*, and the treatment of shigellosis becomes complicated (DeRoeck et al., 2005). Sulphonamide was the first to treat shigellosis, followed by chloramphenicol and tetracycline. When *Shigella* established resistance against all those antimicrobial agents, the treatment was moved to co-trimoxazole and ampicillin. *Shigella* developed resistance again to the previous drug; thus, treatment turned to nalidixic acid. The following resistance capacity to nalidixic acid introduced the next antimicrobial agent to *Shigella*, fluoroquinolones. However, isolation from various sources currently shows the resistant strains of *Shigella* to fluoroquinolones (Puzari et al., 2018). Ceftriaxone, pivmecillinam, and azithromycin were the antimicrobial agents used to replace fluoroquinolones to treat the infection (Taneja & Mewara, 2016). Unfortunately, isolates are resistant to ceftriaxone and azithromycin (Rahman et al., 2007).

This multi-drug-resistant *Shigella* has become a therapeutic challenge and a considerable concern worldwide. Therefore, innovative strategies are needed to overcome the global impact of shigellosis as the existing prevention and treatment measures are inadequate to control the incidence. Several approaches to prevent this infectious disease are generalizable, such as improving water and cleanliness and basic hygiene practices (Asbury et al., 2018). Other than that, there could be a generous benefit to developing a vaccine versus the most prevalent serotypes of *Shigella* (Niyogi, 2005). Before the vaccine development, a comprehensive understanding related to the disease-causing mechanisms of *Shigella* is essential to assist in this vaccine development (Jennison & Verma, 2004).

2.1.6 Pathogenesis of shigellosis

All serotypes of *Shigella* follow a similar pathogenesis pathway. *Shigella* have neither flagella nor adherence factor. The primary basis of the pathogenesis of

shigellosis is the ability of the bacteria to penetrate and reproduce in the intestinal epithelium, resulting in epithelial inflammation and destruction (Niyogi, 2005). *Shigella* has developed several strategies to escape from being destroyed by the immune cells and invading the neighboring cells (Ismail et al., 2002). The epithelial cells of the intestine act as a barrier to prevent the pathogen in the lumen of the gut from entering the epithelial layers. Unfortunately, *Shigella* can penetrate the colonic epithelial layers and access the lamina propria (Jennison & Verma, 2004). They can invade the intestinal epithelium by secreting virulent factors into the host cell through a type III secretion system, resulting in the down-regulation of the expression of active components, cathelicidin, and beta-defensin hence regulating innate signaling (Ashida et al., 2015). Besides, it has been reported that *Shigella* can turn off the endogenous antimicrobial proteins expression, the essential effector molecules of innate immunity expressed in phagocytes and at epithelial surfaces, resulting in severe shigellosis infection (Gudmundsson et al., 2010).

The *Shigella* infection involves multiple steps that start with the translocation through a particular epithelial cell called microfold cells (M cells). As showed in Figure 2.4, the M cells can carry the whole body of *Shigella* from the intestinal lumen to the underlying mucosal layer through transcytosis. After the transcytosis, the resident macrophages engulf *Shigella* by phagocytosis (Sansonetti and Phalipon, 1999). However, instead of killing the bacteria by phagosome, the *Shigella* can escape the phagosome through invasion plasmid antigens (Ipa) B-mediated phagocytic vacuole lysis (High et al., 1992). This IpaB attaches and stimulates pro-apoptotic cysteine proteases, causing the macrophages to undergo apoptosis (Chen et al., 1996).

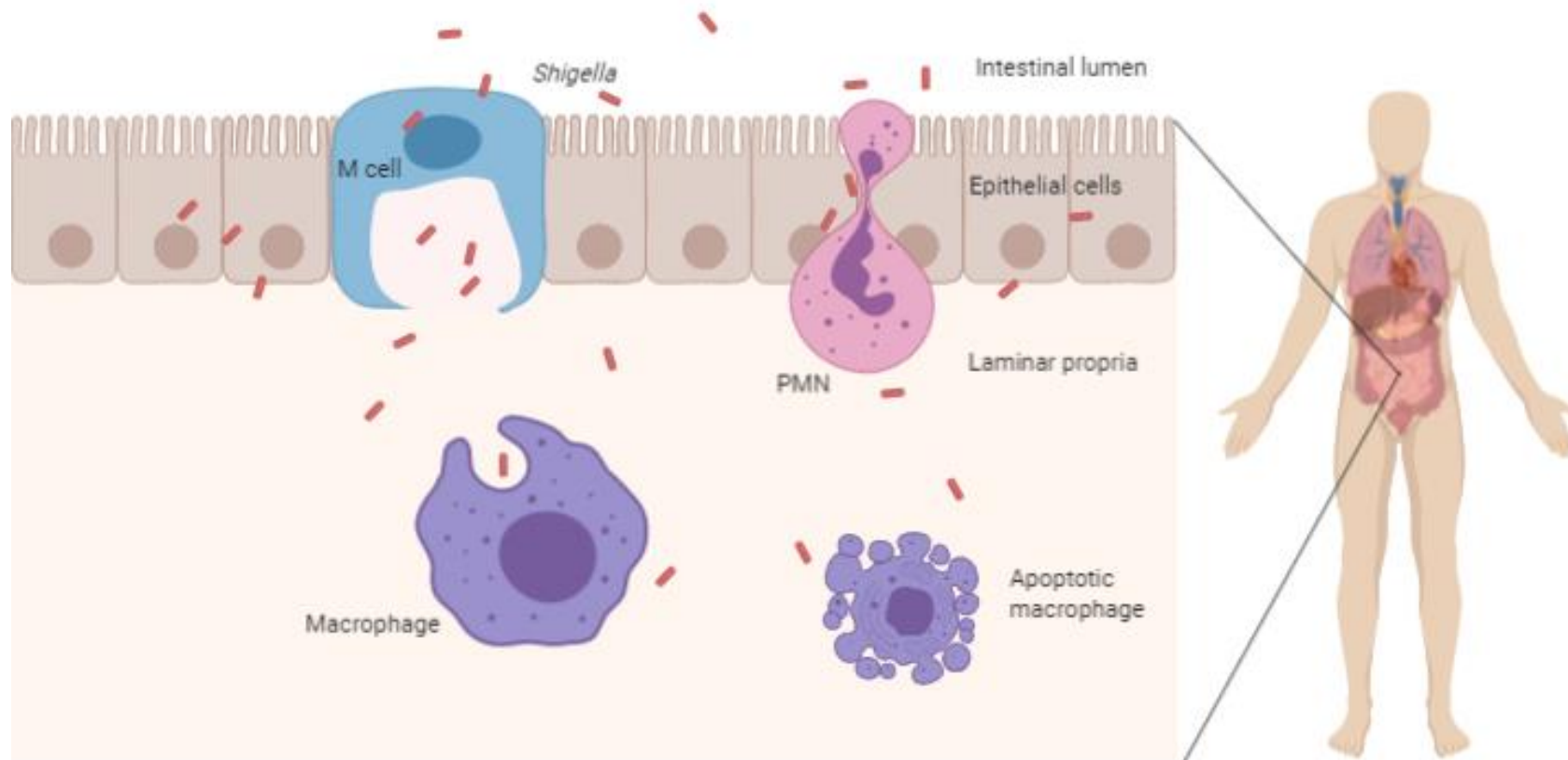


Figure 2.4: *Shigella* invasion during shigellosis. The process begins when *Shigella* invades the epithelial barrier through M cells, followed by the uptake by the resident macrophages. However, *Shigella* produce a variety of virulent factors to escape the phagosome and induce macrophage apoptosis. The apoptotic macrophages produce cytokines, which leads to the recruitment of PMN cells. This causes the destabilization of the epithelial layer, subsequently allowing the invasion of more *Shigella* through a basolateral route and dissemination within the mucosa. The figure was created with BioRender.com.

During apoptosis, the macrophage cell death is attended with the secretion of a large number of cytokines, including interleukin (IL)-1 β and IL-8, which is mainly to recruit polymorphonuclear (PMN) cells to the infection site (Beatty & Sansonetti, 1997). PMN recruitment compromises the integrity of the epithelial layer, which triggers the destabilization of the epithelial layer, subsequently permitting the invasion of *Shigella* to intestinal epithelial cells through a basolateral route (Carneiro et al., 2008). In this early stage of infection, the tissue alteration facilitates impaired adsorption of water, nutrients, and solutes that subsequently causes watery diarrhea (Niyogi, 2005).

Once internalized, *Shigella* begins to rapidly reproduce while engaging the host actin to drive the *Shigella* within the intracellular host environment. This allows efficient intracellular movement and accelerates cell dissemination (Schroeder & Hilbi, 2008). The characteristic of *Shigella* pathogenesis that makes it particularly detrimental to infect an individual is the rapid spreading of the bacteria via the epithelial layer without the requirement to re-enter the basolateral pocket to infect the neighboring cells (Schroeder & Hilbi, 2008). Consequently, *Shigella* can spread and replicate within the epithelial layer of the intestine while escaping exposure to the immune cells and extracellular environment (Jennison & Verma, 2004).

2.2 Immune responses against shigellosis

The hallmark of shigellosis is stimulating the host immune response to combat the *Shigella*. The immune system of a host is divided into innate and adaptive immunity. The interaction of bacteria with the mucosal epithelial cells induces multiple processes that start with innate immune recognition of the microbial pathogen. Briefly, the innate immune response indirectly alerts the immune cells of

the host to the infection. It ultimately triggers the adaptive immune response, mediated by T-cells and B-cells. This specific immunity is responsible for clearing the infection and developing memory components of the disease (Janeway & Medzhitov, 2002).

2.2.1 Innate immunity

The first line of defense against *Shigella* infection is the mucosal intestinal epithelial layer of the host (McGhee & Fujihashi, 2012). This epithelial barrier protects the host from the invasion and systemic spread of commensal and pathogenic microorganisms (Phalipon & Sansonetti, 2007). The viscosity, thickness, and high acidity of this mucosa protect the host by preventing microbial adherence and interpenetration (Silva et al., 2013). However, one study proved that *Shigella* could survive in the stomach with high acidity better than other enteric pathogens. The *Shigella* isolates could survive at pH 2.5 for more than two hours, while not a single *Salmonella* isolate can survive (Gorden & Small, 1993).

Antimicrobial proteins in the intestinal environment are essential in the bacterial host defense (Hancock & Scott, 2000). Antimicrobial proteins are lytic enzymes that disrupt microbial membrane structure and function (Ganz, 1999). The primary classes of antimicrobial proteins in humans are cathelicidins and defensins. A report about the expression of human intestinal defensin in transgenic mice proved the importance of this defensin as an intestinally-secreted antibiotic peptide to attack the infection from *Salmonella typhimurium* (Salzman et al., 2003). However, *Shigella* develop a strategy to inhibit the generation of these antimicrobials (Phalipon & Sansonetti, 2007).

Once *Shigella* have invaded the epithelial barrier of the host, body defense depends initially on innate or nonspecific mechanisms (Nauciel, 1999). In the early defense, the innate immune response involves immune cells against foreign