Gene Expression Profiles of Vesicant-Induced Skin Injury

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ABSTRACT

Cutaneous exposure to sulfur mustard [bis-(2-chloroethyl)-sulfide; SM, HD] results in characteristic blister formation in the exposed skin. The blister is not immediate, but develops within the first 24 hours after SM exposure. During this period there is a marked inflammatory response that involves a variety of cell types including neutrophils, macrophages, T-cells, B-cells, and fibroblasts. Skin from mice exposed to a high level (0.08 mg) of SM was collected at early (6, 12, and 24 h post exposure) or late (72 and 168 h post exposure) time intervals, the RNA isolated, and analyzed for gene expression analysis by the Affymetrix Murine high-density oligonucleotide array (46K genes and ESTs). Expression of more than 1000 independent mRNAs was upregulated greater than 2-fold in a minimum of one of the five timepoints in SM-exposed skin. Data was organized into four major groups based on cluster analysis: 1) those mRNAs with a 2-fold increase beginning within 24 h and persisting throughout the study; 2) those mRNAs with a 2-fold increase within 24 h that became down-regulated in at least one of the later timepoints; 3) those mRNAs with a 2-fold increase starting at 72 h; 4) those mRNAs with a 2-fold increase starting at 168 hr. The mRNAs induced by sulfur mustard were organized into biological functions such as transcription factors, inflammatory factors, biosynthesis molecules, inducers of apoptosis, etc. Selected mRNAs from each category were analyzed by quantitative RT-PCR for microarray verification and to identify potential biomarkers of chemical injury. The results provide a further understanding of cutaneous SM exposure and identify other potential diagnostic markers of and therapeutic targets for treating exposure to SM.

1.0   INTRODUCTION

The chemical warfare agent, sulfur mustard (bis[2-chloroethyl] sulfide, SM, HD) was used as recently as 1988 in the Iran/Iraq. Sulfur mustard penetrates the skin and causes extensive blistering after a latent period of several hours (1). The resultant skin damage includes inflammation, epithelial tissue damage, and damage to the anchoring complex of the dermal-epidermal junction (DEJ), culminating in the formation of subepidermal blisters (1, 2). A variety of molecules are upregulated after exposure, many of them involved in wounding and repair mechanisms. In order to identify potential and specific biomarkers of time-induced sulfur mustard injury, mouse ear punch biopsies were collected at various timepoints after SM exposure and microarray

analysis performed. Those mRNA's that increased at least two-fold with P-values of <0.05 were selected by cluster analysis and sorted according to biologic function. A number of mRNAs representative of a variety of biologic functions was used for RT-PCR to confirm the microarray data.

2.0   MATERIALS AND METHODS

Animal exposures and tissue processing. Exposures to SM were carried out in Battelle's Biomedical Research Center, West Jefferson, OH, USA, which contains chemical agent laboratories that are Department of Army approved. Male Crl:CD1 (ICR) BR mice (Charles River Laboratories, Portage, MI, USA), weighing 25 to 35 g, were anesthetized prior to SM exposure using i.p. administered ketamine hydrochloride (100 to 200 mg/kg) and xylazine hydrochloride (3 to 5 mg/kg). For SM exposure, 5 μl of 97.5 mM SM (0.08 mg) diluted in CH_2Cl_2 (treated) was applied to the inner medial surface of the right ear and 5 μl or CH_2Cl_2 (control) to the inner medial surface of the left ear, as previously described (Casillas *et al.*, 1997). For the microarray analysis 3 separate animals were taken at 6, 12, 24, 72 and 168 h after exposure. The ears were not decontaminated following exposure. In addition, tissues were taken from n = 3 ears of untreated mice (naïve control). After exposure, animals were placed in polycarbonate cages within a chemical fume hood with food and water provided ad libitum. Animals were euthanized (halothane overdose) within the fume hood and both ears were removed using a pair of sharp scissors. An 8-mm tissue biopsy punch was used to remove a full-thickness skin biopsy from the center of the exposed and control ears and used for RNA preparation.

RNA isolation and reverse transcription. Total RNA was isolated from the frozen ear punches using TRIzol® reagent, according to the manufacturer's instructions (Invitrogen Corporation, Carlsbad, CA, USA). Eppendorf Phase Lock Gel® (Brinkmann Instruments, Westbury, NY, USA), a product that eliminates interface-protein contamination during phenol extraction, was added during centrifugation. The RNA pellet was dissolved in RNA Storage Solution® (Ambion, Austin, TX, USA), and RNA was quantitated spectrophometerically based on absorbance at 260 nm. Total RNA (1 μg) was reverse-transcribed into cDNA using a First-Strand Synthesis System® (Invitrogen) for reverse transcriptase-polymerase chain reaction (RT-PCR). A minus reserves transcriptase reaction was included as a control.

Gene expression with microarrays. Between 5 and 20ng of total RNA from each sample was used to generate a high fidelity cDNA for array hybridization. Ribo-SPIA™ linear amplification process was used to generate "antisense" cDNA by DNA polymerase replication of a double stranded DNA template prepared from mRNA. Single strand cDNA was prepared from total RNA using a unique DNA/RNA chimeric primer and dreverse transcriptase. The resulting structure is double stranded DNA with a RNA/DNA heteroduplex of unique sequence at one end. DNA polymerase and RNAse H were used to amplify the DNA and remove the RNA. Following cDNA amplification the product is fragmented (50-100bp) and labeled with biotin in preparation for hybridization to Affymetrix GeneChips. All samples were subjected to gene expression analysis via the Affymetrix mouse 430_2.0 Plus high-density oligonucleotide array, which currently queries 46,000 mouse probe sets. Hybridization, staining and washing of all arrays were performed in the Affymetrix fluidics module as per the manufacturer's protocol. Streptavidin phycoerythrin stain (SAPE, Molecular Probes) was the fluorescent conjugate used to detect hybridized target sequences. The detection and quantitation of target hybridization was performed with a GeneArray Scanner 3000 set to scan each array twice at a factory set PMT level and resolution.

Data Normalization and Cluster Analysis. The Microarray Analysis Suite 5.0 (Affymetrix) was employed to interpret the comparative analysis using distinct algorithms to determine the presence or absence of a

transcript, the differential change in gene expression [increase (I), decrease (D), marginal increase (MI), marginal decrease (MD), and no change (NC)], and the magnitude of change, which is represented as signal log ratio (on a log base 2 scale). T-tests were performed on the normalized signal values prior to exploring additional analyses. All data presented were from pairwise comparison analysis. Iobion's GeneTraffic was used to perform Robust Multi-Chip Analysis (RMA), which is a median polishing algorithm used in conjunction with both background subtraction and quantile normalization approaches. This approach minimizes the inherent noise in GeneChip data, which enhances the discriminating power of the analysis. Hierarchical clustering analyses were applied to the data sets.

Real-time polymerase chain reaction. All genes selected from the microarray data set for validation will be analyzed as described below. Probe and primers were designed through online Universal Probe Library Assay Design ProbeFinder 2.20. Forty nanograms of total RNA from each sample was used to generate a high fidelity cDNA for QPCR analysis using NuGen Ovation Biotin RNA Amplification and Labeling system (NuGen, Cat#4200-60). QPCR was performed on an ABI Prism 7900 (Applied Biosystem), using 2XABI Universal Master Mix (Applied Biosystem) and with sets of primers and Univeral ProbeLibrary Probes (Roche) designed online with ProbeFinder version 2.2 (Roche). All reactions were performed in triplicate and with the following cycle parameters: 1 cycle of $50^{o}C$ (2 min) followed by $95^{o}C$ (10 min), 40 cycles of $95^{o}C$ (15 sec) followed by $60^{o}C$ (1min). Data were collected at every temperature phase during each cycle. Raw data will be analyzed using the Sequence Detection Software (ABI, Foster City CA) while relative quantitation using the comparative threshold cycle (C_T) method will be performed in Microsoft Excel (ABI Technote #2: Relative Gene Expression Quantitation). The mRNA levels were measured from samples taken at 6, 24, 72, and 168 h by real-time RT-PCR. Data (three biological repeats and three technical repeats at each time point) were expressed as fold change relative to CH_2Cl_2 control, and the fold changes in RNA between the SM-treated and control at each time were analyzed statistically.

Statistics. Data were reported as mean ± SE. For the RT-PCR experiments, data were analyzed for log-transformed values of RNA using ANOVA and SAS® programmable language (Littel *et al.*, 1996). To determine if significance levels changed after multiple testing, a Bonferroni correction was applied. A p-value of ≤0.05 was defined as significant.

3.0   RESULTS

The isolated RNA from skin of mice exposed to SM was collected at five timepoints (6, 12, 24, 72 and 168 h post exposure), and analyzed for gene expression analysis by the Affymetrix Murine high-density oligonucleotide array GeneChips (46K genes and ESTs). Expression of more than 1000 probes was upregulated greater than 2-fold (ANOVA p-values ≤0.05) in SM-exposed tissue compared to control skin for a minimum of at least one of the five timepoints. Data was organized into four major groups based on cluster analysis, which is presented in figures 1 and 2. The analysis identified the following statistically significant groups: 1) those with a 2-fold increase in early (6, 12, 24 h) probes that stayed upregulated through at least one of the late timepoints (72 or 168 h); 2) those with a 2-fold increase in early genes that became down-regulated in at least one of the late timepoints; and 3) those with a 2-fold increase beginning at 72 h; and 4) those with a 2-fold increase beginning at 168 h. Group 1 had 254 gene probes represented; group 2 had 357 gene probes represented; group 3 had 327 gene probes represented; group 4 had 229 gene probes represented.
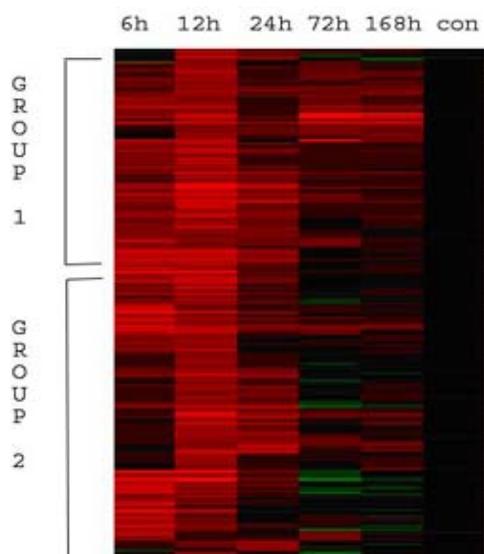
Figure 1: Heat map showing different expression of probes with time after treatment. Probes in red were increased a minimum of twofold (P≤0.05) while transcripts in green were decreased expression. Probes with unchanged expression were colored black. Two major clusters are apparent: group 1 clustered across all the timepoints and group 2 clustered for the early timepoints, but decreased in later timepoints.
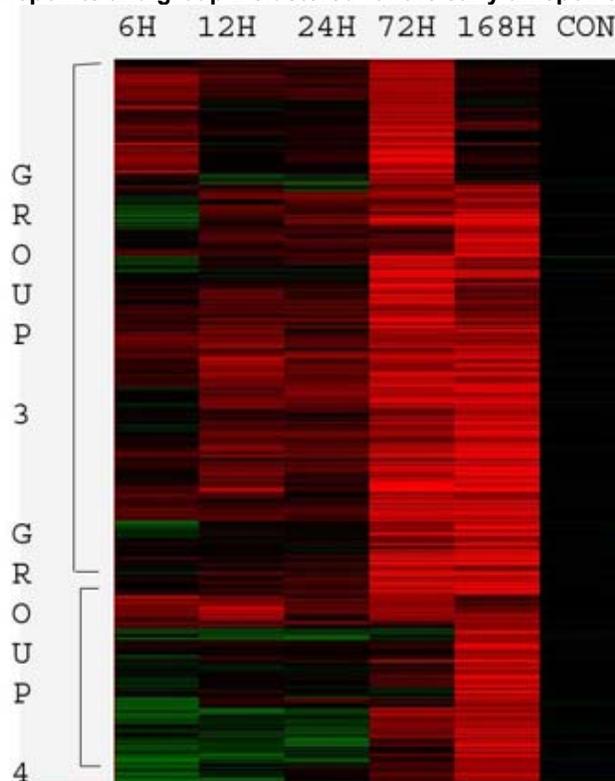
Figure 2: Heat map showing different expression of probes with time after treatment. Probes in red were increased a minimum of twofold (P≤0.05) while transcripts in green were decreased expression. Probes with unchanged expression were colored black. Two major clusters are apparent: group 3 clustered around the 72h timepoint and group 4 clustered around the 168h timepoint.

The gene symbols for each of the groups identified by cluster analysis are shown in Tables 1, 2, 3, and 4.

Table 1: Group 1 Early Genes Upregulated 2x or More and Stay Upregulated

Adam4	Cd24a	Dtr	Hba-a1	Mcam	Pcdh21	Sat1	Stk17b
Adam8	Cd44	Dusp6	Hbb-y	Mcl1	Pdcd1lg1	Scoc	Stx11
Adamts4	Cd53	Eda2r	Hbs1l	Mdm2	Phlda1	Sell	Tcfec
Adm	Cd68	Efhd2	Hck	Mlp	Pira1	Senp5	Tes
Akr1b8	Ceacam10	Emilin2	Hdc	Mmp10	Piwil2	Sh3bgrl	Thbd
Aqp3	Chi3l3	Entpd7	Hif1a	Mmp19	Plac8	Sirpb	Thoc4
Areg	Cias1	Epb7.2	Hist1h2bc	Mmp9	Plaur	Sla	Timp1
Arg2	Clecsf10	Epgn	Hoxc8	Mnda	Plek	Slc11a1	Tiparp
Arl8	Clecsf12	Etv4	Hp	Mpeg1	Plf	Slc15a3	Tlr2
Atf3	Clecsf6	Fcgr1	Hsp105	Mphosph10	Plk2	Slc2a3	Tmprss2
B3gnt1	Clecsf8	Fcgr2b	Hspa5	Mrgpra2	Pmaip1	Slc39a14	Tmsb10
Basp1	Clecsf9	Fgr	Hspa8	Ms4a11s4	Prg1	Slfn1	Tnfaip3
Bcl2a1d	Cmah	Fos	Icam1	Ms4a4c	Procr	Slfn2	Tnfaip6
Bcl3	Coro1a	Fosl1	Ier3	Mt1	Prss18	Slfn4	Trem1
Bst1	Crem	Foxf1a	Ifitm1	Mt2	Pscdbp	Slpi	Trib1
C3ar1	Csng	Fpr1	Il1b	Nap1l1	Psg23	Smox	Trim30
Casp4	Cspg2	Fpr-rs2	Il1rn	Ncam2	Ptgs2	Socs3	Trp53inp1
Ccl12	Ctla2a	Gabrb1	Il4ra	Ncf2	Ptprc	Socs4	Uap1
Ccl2	Ctsz	Gadd45a	Il6	Ncf4	Ptprj	Sox11	Umpk
Ccl3	Cul4a	Gadd45b	Il8rb	Nfil3	Ptx3	Sprr1a	V1rd4
Ccl6	Cxcl1	Gbp2	Itgb2	Nfkbia	Rab20	Sprr1b	Wfdc12
Ccl7	Cxcl2	Gda	Krt1-16	Ngfb	Rad18	Sprr2a	Xdh
Ccl9	Cxcl5	Gjb2	Krt1-17	Nr4a2	Rhob	Sprr2d	Zbp1
Ccng1	Cxcl7	Gjb6	Krt2-6a	Nr4a3	Rnd1	Sprr2f	Zbtb10
Ccr1	Cxcr4	Glipr2	Krt2-6b	Nts	Rps15a	Sprr2h	Zdhhc2
Ccr2	Cyr61	Gm1960	Lcn2	Odc1	S100a8	Sprr2j	Zfp365
Ccr5	D7Ertd458e	Gmfb	Lcp1	Olr1	S100a9	Sprrl1	
Ccrn4l	Ddit4	Gmfg	Lif	Osm	Saa1	Srrm1	
Cct4	Defb3	Gpr171	Lilrb4	Osmr	Saa2	Ssty2	
Cd14	Dio2	Gpr85	Lyn	Ott	Saa3	Stfa1	
Cd244	Dpep2	Has2	Mad	Pabpc1	Samhd1	Stfa3	

Table 2: Group 2 Early Genes Upregulated 2x or More and Then Downregulated

Aaas	Cebpd	Eif2s3x	Hnrpab	Msr1	Pnrc2	Rpo2tc1	Tagap1
Abcb1b	Cebpz	Eif3s1	Hs1bp1	Mt1	Pogz	Rps15a	Takrp
Actb	Cflar	Eif3s8	Hsp105	Mtpn	Polg2	Rps18	Tde1
Adrb2	Cgref1	Eif4a1	Hspa1a	Myc	Polr2a	Rps20	Tex292
Ahcy	Chka	Eif4g2	Hspa9a	Myo1h	Polr3f	Rps25	Tgif
Akap12	Cited2	Eif5	Idb4	Myst2	Ppp1r10	Rrs1	Thumpd1
Alkbh	Clp1	Elys	Ier5	Nap1l1	Ppp1r15b	Rtn4	Tial1
Amd1	Commd3	Enc1	Ifi203	Nars	Prkx	Saa2	Timm13a
Apom	Copeb	Entpd7	Ifitm3	Ncl	Prps1	Sap18	Timm8a
Aqp3	Copg2as2	Epb7.2	Igfbp3	Neu2	Psmd7	Sas	Tmem14c
Arc	Cpne8	Epc1	Impdh2	Nfe2l2	Ptgds	Sdad1	Tnfrsf10b
Arf4	Cpsf2	Epm2aip1	Ipo7	Nfe2l3	Ptger2	Selk	Tnfrsf12a
Arid5a	Cpt1c	Ercc5	Isg20	Nol5	Ptges	Sertad1	Tnfrsf18
Arl10b	Crsp9	Ereg	Jun	Nol8	Ptp4a1	Sf3b3	Tnfrsf6
Arl8	Cspg2	Erh	Kcnq1ot1	Nola1	Rab14	Sfpq	Tnni1
Aspn	Ctps	Etf1	Kctd10	Nolc1	Rad23b	Sfrs6	Tob2
Atf4	Cuzd1	Fgfbp1	Kif5b	Npm1	Ralb	Sfrs8	Top2a
Avpi1	Cycs	Fosb	Klra8	Npm3	Ran	Sirt1	Tpbpb
Axot	Cyp3a13	Gadd45b	Krt1-18	Npn3	Rap2b	Skb1	Tpp2
Bcl10	Daf1	Galnt3	Lats2	Nr2c2	Rbm3	Slbp	Trib1
Bcl2a1a	Dclre1b	Gas5	Lipg	Nr4a1	Rbm8	Slc10a1	Trp53inp1
Becn1	Ddit3	Gcc1	Lox	Nr4a2	Rbmxrt	Slc19a2	Trpm7
Bglap2	Ddx18	Gch1	Lphn3	Nrbf1	Rbx1	Slc25a25	Tslp
Blcap	Ddx21	Gem	Lyar	Nrip1	Reprimo	Slc25a3	Twistnb
Bmp2	Defb1	Gfer	Mad	Nsep1	Rgs2	Slc29a2	Txnrd1
Brca2	Defb5	Gna13	Maff	Oazin	Rhoe	Slc6a14	U2af1
Brd2	Degs	Gnpnat1	Mafk	Odc1	Riok2	Snapc5	Ube2n
Btg2	Dnajb9	Gpr3	Malt1	Orc2l	Ris2	Snrpb2	Ubxd2
Bzw1	Dsip1	Grp58	Mat2a	Orm1	Rnf149	Socs3	Wdr12
C1qbp	Dusp1	Grwd1	Matr3	Pa2g4	Rnf4	Socs4	Wdr40a
Carhsp1	Dusp7	Gtf2b	Mbnl1	Paf53	Rnmt	Socs6	Wdr40b
Ccl20	Eaf1	Gtpbp4	Mdm2	Pde4b	Rnu22	Sprr3	Wig1
Ccnl1	Ebna1bp2	H2-D1	Mftc	Peli1	Rpl12	Ssfa1	Ythdf1
Ccnt1	Eed	H3f3b	Mgea5	Pfdn2	Rpl13a	St7l	Ythdf2
Ccnt2	Eef1e1	Hars2	Mib1	Pglyrp1	Rpl3	Stk17b	Za20d2
Cct3	Ehf	Herc2	Mid1	Phlda3	Rpl35	Strap	Zdhhc13
Cct8	Ei24	Hig1	Mki67ip	Pigf	Rpl36al	Styx	Zfp295
Cd3e	Eif1a	Hist1h2bc	Mrp63	Pigl	Rpl41	Sui1-rs1	Zfp36l1
Cdca4	Eif1ay	Hist1h4a	Mrpl51	Pnp	Rpl5	Taf15	Zfy2

| Cdk5r1 | Eif2s2 | Hnrpa1 | Ms4a10 | Pnpt1 | Rpl7l1 | Taf7 | Zwint |
| Cdkn1a | | | | | | | |

Group 3 Upregulated 2x or More at 72 Hours

Abcg1	Cd80	Dspg3	Gpr65	Lmnb1	Pfn1	Serpina3n	Thrap1
Actb	Cd84	Dyx1c1	Gpsm3	Lrg1	Pgf	Serpine1	Tlr1
Adk	Centa2	Ednrb	Gria2	Lrrtm4	Pgk1	Serpine2	Tlr13
Adn	Chi3l1	Efemp2	Gria3	Lst1	Picalm	Setdb1	Tlr4
Aif1	Clecsf6	Egln3	Grik1	Mamdc1	Pik3ap1	Siat8d	Tlr7
Akr1d1	Clic4	Ehd1	Grm5	Mgl1	Pirb	Six1	Tm6sf1
Akt3	Cntnap2	Ehd2	H19	Mirn142	Pkm2	Slc13a3	Tmc1
Alcam	Col19a1	Elavl2	H2-K1	Mlze	Pla2g7	Slc16a3	Tmem2
Aldh1a3	Cotl1	Emr1	H2-L/D1	Mmp12	Plekhb2	Slc28a2	Tmem7
Angptl4	Cpxm1	Enah	H60	Mmp3	Plod2	Slc2a1	Tmem8
Ankrd1	Crabp1	Eno1	Hao1	Mrc1	Plscr1	Slc2a3	Tnfaip9
Anxa3	Cryab	Eno2	Has2	Ms4a1	Plscr2	Slc7a11	Tnfrsf13b
Apobec1	Csf2rb2	Entpd1	Hemp1	Ms4a4b	Ppp1r3b	Slc7a12	Tnfrsf1b
Apobec3	Csf3	Epha7	Hmga2	Ms4a4d	Prkr	Slc7a8	Tnfrsf22
Arg1	Csprs	Epsti1	Hmox1	Ms4a6b	Prrx1	Slfn3/4	Tnfrsf9
Atbf1	Ctla2a/2b	Ero1l	Hoxa10	Ms4a6c	Prss19	Slfn8	Tnfsf11
Bcl2l11	Ctsb	Esd	Ian4	Ms4a7	Psg28	Smc2l1	Tnfsf9
Bhlhb2	Ctsc	Ets1	Icsbp1	Msn	Ptges	Smr2	Tra1
Bicd1	Ctsl	Fcer1g	Ide	Mup1	Ptgs2	Sn	Treml4
Bnip3	Ctss	Fcgr2b	Ifi47	Myh9	Ptpns1	Snx10	Ttc14
C1qa	Ctsz	Fcrl3	Ifit1	Myocd	Pvt1	Snx5	Tyrobp
C1qb	Cul4a	Fmn2	Igk-V8	Myt1	Rarres2	Snx8	U90926
C1s	Cxcl11	Fosl2	Igsf7	Nav3	Rasgef1a	Sorl1	Uap1
C3	Cxcl12	Foxp2	Il1rl1	Ncf1	Rbp1	Spata5	V1ra1
C3ar1	Cxcl13	Ftl1	Il2rg	Ncoa6ip	Rbp2	Speer7-ps1	V1rc7
Cadps2	Cxcl14	Fxyd4	Inpp5d	Ndrl	Rnasel	Spp1	V2r4
Cald1	Cxcl4	Fxyd5	Itgav	Nedd4l	Rnf134	Sprr2a	Vav1
Capn8	Cyba	Fyb	Itgb1	Nedl1	Rod1	Stab1	Vcam1
Car2	Cyp4f18	Gabrg1	Jundm2	Nek6	Rpl7	Stard8	Vegfa
Car9	Cyp7b1	Gbp4	Kcnd2	Neurod4	Rptn	Steap	Xpo7
Cbr2	Dab2	Gca	Kctd11	Nudt4	Runx1	Syk	Zfp352
Ccl4	Ddah1	Gla	Kif11	Nxph1	Samsn1	T2bp	Zfp617
Ccl8	Defb4	Glrx1	Klra18	P2ry10	Satb1	Tbx3	Zfy2
Ccrl2	Dock2	Gmfg	Klra2	P4ha1	Sdc3	Tcf23	Zic1
Cd163	Dppa2	Gng2	Laptm5	Pcdh10	Sdcbp	Tfpi2	

Cd38	Dppa5	Gp38	Lbh	Pdk4	Sec8l1	Tfrc
Cd47	Dpysl3	Gpr109b	Lcp2	Pfc	Selp	Tgfa
Cd52	Dsc2	Gpr35	Lgmn	Pfkfb3	Serf1	Thbs1

Group 4 Upregulated 2x or More at 168 Hours

Ada	C4	Dmp1	Glrx1	Il10ra	Ly96	Pscd4	Slc7a7
Adam12	Cald1	Dock10	Gnpda1	Il13ra2	Marcks	Psmb8	Snx5
Adamts5	Casp1	Dsc2	Grem1	Il1f6	Masp1	Ptk9	Socs5
Aoah	Casp8	Edg3	Gsto1	Il1f9	Mcpt8	Ptpro	Sp100
Ap1s2	Ccl11	Efemp1	Gusb	Il1rl1	Mefv	Ptprv	St18
Ap2a2	Ccl17	Elk3	Gzma	Il24	Mpn	Pvt1	Stat1
Apob48r	Cd200r1	Entpd3	H2-DMb1	Il2ra	Ms4a7	Rab32	Tcirg1
Aps	Cd48	Esm1	H2-Q7	Il2rb	Msr1	Rab3il1	Tcn2
Arhgap4	Cd69	Ets1	H6pd	Il7r	Msr2	Rassf2	Tcra
Arhgap9	Cd83	Evi2a	Havcr2	Irf1	Mthfd2	Reg1	Tgfb1
Arhgdib	Cd86	Evi2b	Hemp1	Itgax	Myo1f	Reg3g	Tgtp
Arrb2	Centb1	Evl	Hexa	Jak2	Nap1l1	Renbp	Tlr4
Arrdc4	Col18a1	Fbln2	Hfe	Jun	Nov	Rgs1	Tm6sf1
Asahl	Cox6b2	Fetub	Hhex	Kitl	Npc2	Rgs10	Tm7sf1
B2m	Csf2ra	Fgf7	Hk3	Klk6	Oas1g	Rp2h	Tnfrsf21
B4galt6	Csprs	Fgr	Hmga2	Klrb1d	Oas2	Runx2	Tnfrsf5
Bax	Ctla2b	Fhl2	Hpse	Kmo	Oas3	Sca1	Traf1
Bcat1	Ctla4	Fli1	Hrb2	Krt2-1	Oasl1	Scamp5	Trps1
Bcl2l11	Ctse	Flrt2	Ifi1	Lair1	Oasl2	Scarb2	Ugcg
Birc1b	Cxcl10	Flt1	Ifi203	Lgals1	Olfm1	Selp	Usp18
Birc1e	Cxcl16	Folr2	Ifi205	Lgals3bp	P2ry6	Selpl	Vav2
Birc3	Cxcl9	Fscn1	Ifi44	Lhfpl2	Pdgfc	Sema4d	Wisp1
Bloc1s2	Cybb	Gapds	Ifit2	Lpxn	Pik3cg	Serpina3g	Wnt5a
Bmp2k	Cyp7b1	Gas6	Ifitm6	Lrrc28	Pip5k2a	Serpinb11	Zc3hdc1
Btk	Dab2	Gcnt1	Igsf6	Ly6e	Pkib	Serpinb9	Zfpn1a1
Btla	Dcxr	Ggtla1	Igtp	Ly75	Pla1a	Sh3bgrl	
C1qg	Ddx24	Glipr1	Ii	Ly86	Polk	Slc5a8	

The significantly significant gene probes identified from figures 2 and 3 were then sorted according to general function. The functional data is shown in Table 5. Some identified gene probes appear in more than one functional category.

Table 5: General Functions for Cluster Analysis Groups

GENERAL CATEGORIES	Group 1 Early/Late	Group 2 Early Only	Group 3 72 h	Group 4 168 h
Apoptosis	9	9	6	13
Biosynthesis	14	18	34	13
Cell Adhesion	8	12	4	4
Cell Cycle	21	24	42	21
DNA/RNA Repair	6	10	11	14
Electron Transport	4	6	7	6
EST Tags/Unknown Functions	58	43	52	39
Inflammation/Immune	40	51	44	30
Miscellaneous	60	68	88	62
Morphogenesis/Repair	17	19	9	9
Protein Synthesis	15	16	19	13
Proteolysis	6	7	7	6
Signal Transduction	19	20	21	7
Transcription/Regulation	13	18	28	17
Transport	11	17	25	7
Total	301	338	397	261

Three specific probes (Table 6) from each group were selected for confirmation by quantitative polymerase chain reaction. The results of the QT-PCR are in Figure 4.

Table 6: Genes selected for QT-PCR

Group	Symbol	Gene Name	Function
1	Msr1	*macrophage scavenger receptor 1*	Inflammation Pathway
1	Myc	*myelocytomatosis oncogene*	Adhesion Signaling Pathways
1	Tslp	*thymic stromal lymphopoietin*	Cytokine for T cell homeostasis
2	Ccl3	*chemokine (C-C motif) ligand 3*	Inflammation Pathway
2	Cxcr4	*chemokine (C-X-C motif) receptor 4*	Inflammation Pathway
2	Fcgr1	*Fc receptor, IgG, high affinity I*	Immune Response
3	Ccl4	*chemokine (C-C motif) ligand 4*	Inflammation Pathway
3	Cd47	*CD47 antigen*	T cell activation/apoptosis
3	Fos	*FBJ osteosarcoma oncogene*	Sig. Transduction/ Transcription
4	Casp8	*caspase 8*	Apoptosis Pathway
4	Il2rb	*interleukin 2 receptor, beta chain*	T cell regulation
4	Pdgfc	*platelet-derived growth factor*	Stimulates Many Cells

Figure 4: Fold Changes for selected QT-PCR samples
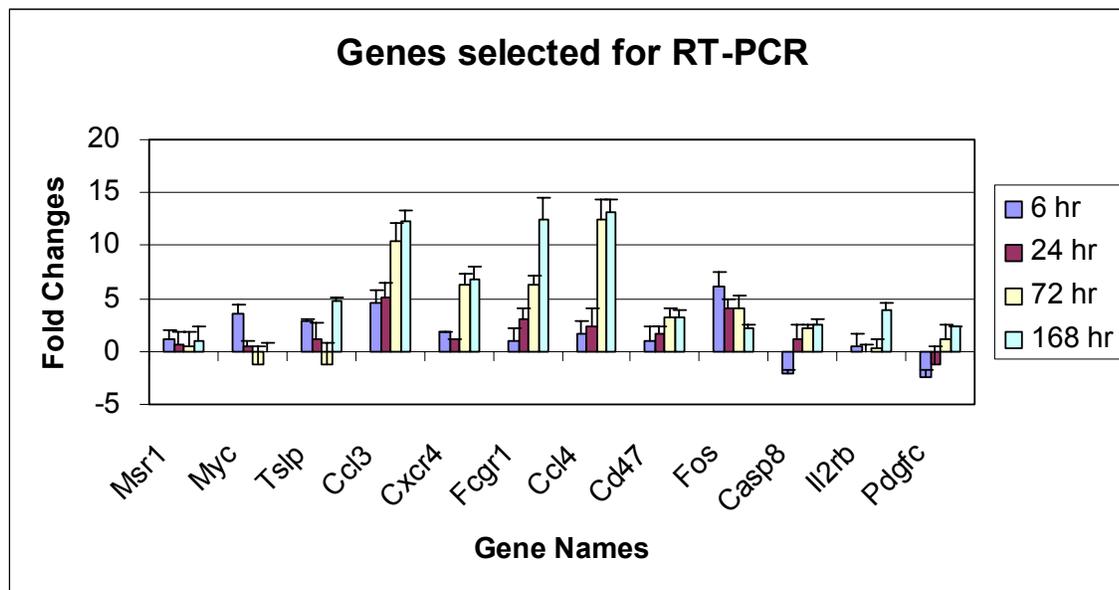
4.0 DISCUSSION

Previous work has examined the gene expression profiles in SM-exposed mouse skin within the first 24h after exposure (3, 4). Since some of the most dramatic pathologic changes (eg blistering) are not early events, the gene expression profiles of later (72h, 168h) timepoints were examined to identify later biomarkers of SM exposure. While a total of 608 genes (Groups 1 and 2) were upregulated ≥2-fold in the early timepoints, an additional 572 genes (Groups 3 and 4) were upregulated ≥2-fold by 168h. Upregulated early genes included genes of inflammation (29 genes) and repair (biosynthesis, 103 genes; DNA/RNA repair, 81 genes; protein synthesis/proteolysis, 58 genes; structural proteins, 78 genes; transcription/regulation, 44 genes). The later genes tended to be in the same categories as the early genes with genes of inflammation (31 genes) and repair (biosynthesis, 142 genes; DNA/RNA repair, 34 genes; protein synthesis/proteolysis, 34 genes; structural proteins, 47; transcription/regulation, 16). The majority of early genes were transcription/regulation and DNA/RNA repair genes. The upregulated later genes were mostly genes of biosynthesis. The normal inflammatory events could be followed by the gene expression patterns. Macrophage secreted factors such as Fcgr1, Il1b, and Il6 are found in group 2 and correspond to macrophage infiltration within the first 24h. Likewise, the B cells infiltrate slightly behind the macrophages and B cell secreted factor genes such as Cd80 and the Tnfrsf family were upregulated at 72 h (group 3). Group 3 also had several complement pathway genes upregulated (C1qa, C1qb, C3). Finally, T cell secreted factor genes Il2ra, Il2rb, Tnfrsf5, and complement pathway genes C1qg, C4, and Masp1 were all upregulated at 168h when an abundance of T cells are expected in damaged tissue. These biomarkers have the potential for identifying a particular stage of wound repair and may be exploited to develop countermeasure treatments in the future.

5.0   REFERENCES

1. Papirmeister B, Feister AJ, Robinson SI, and Ford RD, *Medical Defense against Mustard Gas: Toxic Mechanisms and Phamacological Implications,* CRC Press, Boca Raton, FL, 1991.
2. Petrali JP and Oglesby-Megee S, Toxicity of mustard gas skin lesions. Microsc Res Tech 37:221-228, 1997.
3. Rogers JV, Young WC, Kiser RC, Babin MC, Casillas RP, Schlager JJ, and Sabourin CLK, Microarray Analysis of Gene Expression in Murine Skin Exposed to Sulfur Mustard, J Biochem Mol Tox 18:289-299, 2004.
4. Dillman III JF, Hege AI, Phillips CS, Orzolek LD, Sylvester AJ, Bossone C, Henemyre-Harris C, Kiser RC, Choi YW, Schlager JJ, and Sabourin CL, Microarray Analysis of Mouse Ear Tissue Exposed to Bis-(2-chloroethyl) Sulfide: Gene Expression Profiles Correlate with Treatment Efficacy and an Established Clinical Endpoint, J Pharm and Exp Therap 317:76-87, 2006.